Hydrothermal pretreatment of oxytetracycline fermentation residue: Removal of oxytetracycline and increasing the potential for anaerobic digestion

Mohammed Awad¹, Zhe Tian^{1,2*}, Yu Zhang^{1,2}, Min Yang^{1,2}, Wenjuan Yin³, Liping Dong³

¹State Key Laboratory of Environmental Aquatic Chemistry, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

²University of Chinese Academy of Sciences, Beijing 100049, China

³State environmental protection engineering center for harmless treatment and resource utilization of antibiotic residues, Xinjiang 835007, China

ABSTRACT

The presence of high level of antibiotics in the antibiotic fermentation residue is one of the main reasons that prevent their direct disposal or further use as a resource. In this study, the feasibility of using the hydrothermal pretreatment for removing oxytetracycline (OTC) from its fermentation residue and enhancing anaerobic digestion was evaluated under different temperatures i.e. 110, 130, 150 and 170°C. The results showed that the removal rate of OTC increased as a function of temperature, and hydrothermal treatment at 130°C for 5 min was found sufficient to reduce the concentration of OTC from 3.9 mg/g to less than the detection limit (i.e., 0.25 ng/g). Biochemical methane potential tests showed that the cumulative methane production over 23 d was 73.7, 215.9, 656.8, and 439.0 NmL CH₄/gVS for the raw residue and the residue treated at 130, 150, and 170°C for 5 min, respectively. At the same time, the abundances of tetracycline resistance genes were reduced by hydrothermal treatments followed by anaerobic digestion. Conclusively, it is suggested that hydrothermal treatment at 150°C for 5 min was found beneficial for OTC fermentation residues ensuring the removal of OTC and further use of the residue for anaerobic digestion.

Keywords: Antibiotic fermentation residue, Biochemical methane potential, Oxytetracycline, Thermal hydrolysis

1. Introduction

Oxytetracycline (OTC) is one of the broad-spectrum antibiotics widely used in livestock farming and aquaculture worldwide. OTC is produced by submerged fermentation of *Streptomyces rimosus* [1] which yields large amounts of OTC fermentation residue at the end of the fermentation process. Because of the high level of residual antibiotics, the antibiotic fermentation residue have been added into the list of hazardous materials [2], and are prohibited to be used as animal feed or fertilizer and their open dumping without proper treatment is also prohibited [3]. At present, incineration is the most commonly used treatment technology for antibiotic fermentative residue, but it is very expensive and energy-intensive due to the high moisture content in the residue, which becomes a great economic burden to the manufacturers [4]. Therefore, it is necessary to explore alternative technology to reduce the treatment cost and transform

the antibiotic fermentation residue into a beneficial material.

Upon successful removal of the antibiotics, the antibiotic fermentation residue can be utilized as carbon-rich biomass [5]. Although, Fenton process, ozonation, and other advanced oxidation processes are found effective for the reduction of OTC [6, 7], they have certain shortcomings including high chemical oxygen demand (COD) and high level of suspended solids in the residue [8]. Since antibiotics are prone to hydrolysis at elevated temperatures, hydrothermal treatment may be a suitable alternative technology for their successful removal from the residue [9]. At present, hydrothermal pretreatment has been applied in the treatment of waste mother liquor from the antibiotic fermentation process [10]. Recently, we reported that hyperthermophilic anaerobic digestion process operated at 70°C for 7 d could simultaneously remove antibiotics, reduce antibiotic resistance genes (ARGs) and enhance the solubility of spiramycin fermentation residue [11]. To further improve efficiency and reduce processing

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* Corresponding author

Email: zhetian@rcees.ac.cn

Tel: +86-010-62843038 Fax: +86-10-62923541

time, the thermal hydrolysis process operated at more than 120°C [12, 13] has been developed. For example, CambiTHP™ and Biothelys®, two commercially available hydrothermal processes with an operation temperature 165°C, have been established as the pretreatment units prior to anaerobic digesters for treating municipal sewage sludge [14]. The hydrothermal treatment has been proved to significantly enhance the solubility of biomass and thus greatly increase biogas production [15, 16]. Recently, Pei *et al.* [16] found that thermal hydrolysis at 170°C for 30 min could effectively reduce the abundance of antibiotic resistance genes in pharmaceutical and municipal waste sludge. The hydrothermal process has also been tested for the treatment of streptomycin and cephalosporin C residues, where an improved biodegradability of the residues with complete removal of the residual antibiotics were proved [17-19]. Considering the facile hydrolysis of OTC at high temperatures [20], hydrothermal treatment can be considered highly desirable for the treatment of OTC fermentation residues, however, its feasibility so far remains unclear.

In the present study, the feasibility of hydrothermal hydrolysis as a pretreatment method of the OTC fermentation residue was evaluated over a temperature range from 110 to 170°C for a period of 5 - 30 min. The effect of temperature on the removal of OTC, reduction of ARGs, and enhancement of solubility were investigated first. Then the biochemical methane potentials (BMP) of the raw and treated residues were tested using Automatic Methane Potential Test System (AMPTS), and the changes of ARGs and microbial communities before and after the BMP test were detected using quantitative PCR analysis and Miseq sequencing. The results of this study provide guidance for the establishment of a viable strategy for the treatment of antibiotic fermentation residues.

2. Materials and Methods

2.1. Experimental Setup

In this study, a high-pressure stainless steel reactor covered with a Teflon liner (effective volume, 1.2 liters) was used for the thermophilic hydrolysis experiment (Weihai Borui Chemical Machinery, China). The OTC residue acquired from an OTC manufacturer in Hebei Province, which mainly produces oxytetracycline dehydrate via fermentation of *Streptomyces rimosus*. The fermentation residue was collected from the production workshop as soon as it was separated from the fermentation broths. The collected residue was stored at 4°C before use, and its characteristics are provided in Table 1. For each test, approximately 0.5L of the residue was

added to the hydrothermal reactor. The hydrothermal treatment was performed at various temperatures (*i.e.*, 110, 130, 150, and 170°C) and for different time periods (5, 10, 15, 25, and 30 min). During the treatment, the biomass was mixed at a speed of 180 ± 5 rpm. Samples for antibiotics analysis were stored at -20°C until use.

2.2. Analytical Methods

In order to characterize the performance of hydrothermal treatment on enhancing solubility of OTC residue, the concentrations of solubilized COD and ammonia nitrogen were measured. As for solubilized COD, the residue was first centrifuged at 10,000 rpm for 10 min and then filtrated using a 0.45µm filter. COD of the filtrate was determined using Spectroquant photometer NOVA 60 (Merck D armstadt, Germany). Ammonia nitrogen was detected using the Nessler reagent spectrophotometric method. Total solids and volatile solids were determined according to standard methods [21].

2.3. Determination of Oxytetracycline in Fermentation Residue

The procedure described by Qiao *et al.* [22] and Zhu *et al.* [23] was used for the analysis of OTC in the fermentation residue with some modifications. Briefly, 0.1 g of Na₂EDTA and 10 mL extraction buffer (phosphate buffer (pH = 3.0) : acetonitrile = 1:1 (v/v)) were added to 50 mg freeze-dried fermentation residue sample. The mixture was vortexed for 30 s and sonicated for 20 min, followed by centrifugation at 3,000 rpm for 20 min, and then the supernatant was decanted into a new glass bottle. This step was repeated twice. The combined supernatant was diluted with distilled water to 500 mL, followed by adding 1 mL phosphate buffer (pH = 3.0). The extract was subjected to solid-phase extraction using Oasis HLB extraction cartridges. The antibiotic fraction eluted from the cartridges was dissolved in 0.5 mL of methanol. Before UPLC-MS/MS analysis, the methanol extracts were diluted at 1:1 (v/v) with pure water. Analysis of the extracts was performed by a Waters ACQUITY UPLC™ system (USA) equipped with a Waters Micromass Quattro Premier XE (triple-quadrupole) detector following Zhu *et al.* [23]. Samples were analyzed in triplicate. Recovery was determined by spiking OTC standard into the 170°C hydrolyzed residue (5 min) at the concentration of 400 µg/kg, followed by the same extraction and determination procedure described above. The average recovery of OTC was 85.6% ± 7.1%.

2.4. BMP Test

In order to compare the performance of different hydrothermal pretreatment conditions for transforming the OTC fermentation residue into beneficial material, the methane formation potentials of the residue with and without hydrothermal pretreatment were tested in duplicate using the Automatic Methane Potential Test System (AMPTS 2.0) (Bioprocess Control, Sweden). Since the hydrothermal treatment at 110°C was not able to completely remove the residual antibiotic (Table 2), it was excluded from the BMP test. The test was performed in a 500 mL glass bottle containing 400 mL of substrate and inoculum at a ratio of 1:1. Before the BMP test, the pH of the residue with or without pretreatment was adjusted to around 7.0. The inoculum was obtained from the mesophilic digester of the Gaobeidian Wastewater Treatment Plant in Beijing (Table S1). The system was operated at mesophilic condition

Table 1. Characteristics of The OTC Fermentation Residue Used in The Experiment

Parameter	Value
pH	5.42 ± 0.0
TSS (g/L)	91.5 ± 1.2
VSS (g/L)	74.8 ± 0.4
VSS/TSS (%)	81.8 ± 0.0
sCOD (mg/L)	9443 ± 233
NH ₃ -N (mg/L)	244 ± 23
OTC content (mg/g)	3.9 ± 0.1

Table 2. Removal of OTC from The Fermentation Residue Under Different Treatment Temperatures and Times

		Concentration of OTC ($\mu\text{g/g}$)				
Time/min		5	10	15	25	30
Temperature ($^{\circ}\text{C}$)	110	29.9 ± 3.5	33.9 ± 3.6	14.9 ± 0.6	14.6 ± 0.8	15.2 ± 0.3
	130	BDL*	BDL	BDL	BDL	BDL
	150	BDL	BDL	BDL	BDL	BDL
	170	BDL	BDL	BDL	BDL	BDL

*BDL: Below Detection Limit ($< 0.25 \text{ ng/g}$)

(37°C) for 23 d, which is often used for anaerobic mesophilic digestion studies [24-26] and gas production was automatically recorded by AMPTS 2.0. The methane formation potential was expressed as the volume of produced methane/g of VS.

2.5. DNA Extraction and Quantitative PCR Analysis

Total DNA was extracted from 0.5 mg of all samples using a FastDNA SPIN kit for soil (MP Bio, USA), according to the manufacturer's instructions. The quality and quantity of the extracted DNA were checked by using NanoDrop and gel electrophoresis. For each sample, bacterial 16S rRNA gene and eight frequently reported tetracycline resistance genes including *tetA*, *tetC*, *tetG*, *tetK*, *tetL*, *tetM*, *tetO*, and *tetX* were examined. Only three *tet* genes namely *tetA*, *tetO*, and *tetX* were successfully detected. The quantification was carried by SYBR green quantitative real-time PCR (qPCR) using LightCycler 96 (Roche Diagnostics GmbH, Germany) as previously described [27]. The amplification was carried out in a 25 μL reaction mixture. All reactions were performed in triplicate. The temperature program was set as one cycle of 95°C for 10 min followed by 40 cycles of 95°C for 15 s and annealing temperature for 1 min. The primers' information and the annealing temperature of different ARGs are shown in Table S2 in the supporting material. Gene abundance was calculated using software supplied with LightCycler. A standard curve was generated using a given concentration of standard plasmids containing each target gene. The correlation coefficients (R^2) for standard curves and amplification efficiencies based on curve slopes were calculated to ensure reliable amplification. The R^2 values for all the curves were over 0.99 and efficiency between 92% and 108%. The specificity of the PCR was also assured by melting curves and gel electrophoresis.

2.6. Miseq Sequencing

Microbial community composition of different samples of the BMP test was analyzed using Illumina MiSeq sequencing. The sequencing was performed using the protocol described by Tian et al. [27]. The DNA samples were used to amplify the V4-V5 hypervariable regions of the 16S rRNA gene using the primers set of 515F/907R [28]. DNA was amplified in triplicate for each sample. PCR amplicons were pooled together and purified by Agarose Gel DNA purification kit (TaKaRa). The sample libraries were pooled, and paired-end sequencing was conducted with an Illumina MiSeq platform (Illumina, USA) according to the standard protocols. Amplicon data analysis including quality filtration, chimeras check, and taxonomic classification were conducted following Tian et al. [27]. The raw reads were deposited into the NCBI Sequence Read Archive

(SRA) database with accession no. PRJNA612953.

2.7. Statistical Analysis

The significant difference of the *tet* genes and other parameters between different treatments was compared using ANOVA (SPSS 22.0, IBM, USA). The p -value was considered statistically significant at $p < 0.05$. Clustering analysis and Heatmap of the bacteria genera in digested sludge after the BMP test were generated using HemI [29].

3. Results and Discussion

3.1. Removal of Antibiotics from OTC Fermentation Residue under Different Hydrothermal Pretreatment Conditions

The residual OTC concentrations under different hydrothermal treatment conditions are presented in Table 2. It is clear that the hydrothermal treatment was quite effective even at a temperature of 110°C with the residual OTC concentration decreasing from $3.9 \pm 0.1 \text{ mg/g}$ to $29.9 \pm 3.5 \mu\text{g/g}$ (removal, 99.2%) for 5 min, and further to $14.9 \pm 0.6 \mu\text{g/g}$ (99.6%) by extending the treatment time to 15 min. However, the residual OTC concentration was not further reduced even by extending the treatment time to 30 min, suggesting that higher temperature was required to allow complete removal of OTC. When the temperature was increased to 130°C , the residual OTC was decreased to below detection limit ($< 0.25 \text{ ng/g}$) after 5 min treatment, indicating that 130°C was optimum temperature threshold for completely removing OTC from the fermentation residue.

There are a few other studies that also focused on the hydrothermal treatment of fermentation residues. For instance, Zhang et al. [17] performed the hydrothermal process for the treatment of two types of cephalosporin C fermentation residues that contain different concentrations of saccharides. They reported that hydrothermal pretreatments at 200 and 180°C for 20 min were sufficient for the complete decomposition of cephalosporin C in low and high saccharide content of antibiotic fermentation residues, respectively. Sun et al. [30] evaluated the hydrothermal pretreatment of sewage sludge at 160°C for 30 min and found decline in tetracycline concentration from 68.6 ng/g to below detection limit (12 ng/g). OTC was found to be easily hydrolyzed both in water and soil when the temperature is higher than 45°C [31, 32]. Recently, Yi et al. [10] reported that the half-life ($t_{1/2}$) of OTC in water at pH 5 and temperature 85°C was just 0.26 h, and an increase of 10°C accelerates the OTC hydrolysis rate by 1.74-5.58 fold. This

study demonstrated that even adsorbed by the fermentation residue, OTC could be completely hydrolyzed at a temperature of 130°C. Meanwhile, the major OTC hydrolysis products were found as 4-epioxytetracycline, α -apo-oxytetracycline and β -apo-oxytetracycline [33], whose potencies are only equivalent to 0.7%, 0.3% and 0.7% of that of the parent compound, respectively [34]. Therefore, the generated OTC hydrolysis products might not put a selection pressure on environmental bacteria for development of antibiotic resistance during the subsequent application of the hydrolyzed residue.

3.2. Solubilization of OTC Fermentation Residue during Hydrothermal Pretreatment

The solubilization effects of the OTC fermentation residue under different hydrothermal treatment conditions were also investigated by monitoring the changes of soluble COD and $\text{NH}_3\text{-N}$. As shown in Fig. 1, the hydrothermal treatment was quite effective in the solubilization of the fermentation residue, resulting in a marked release of soluble organic substances and $\text{NH}_3\text{-N}$. The soluble COD increased from 9,443 mg/L to 29,730 mg/L at a temperature of 110°C for 5 min. Extension of treatment time did not significantly affect the solubilization effects of the fermentation residue. Similar to the OTC removal, elevating the temperature to 130°C led to the increase of soluble COD to 47,072 mg/L, however further elevating treatment temperature did not lead to a perceptible increase in soluble COD concentration. $\text{NH}_3\text{-N}$ release on the other hand was dramatically increased with the increase of temperature, but was found indifferent to treatment time variation. The concentration of $\text{NH}_3\text{-N}$ was increased from 244 mg/L in raw residue to 684, 781 and 798 mg/L for the samples pretreated at 110, 130 and 150°C, respectively. On further increase to 170°C, the concentration of $\text{NH}_3\text{-N}$ was increased to 976.6 mg/L. It is possible that the released proteins were further hydrolyzed to release $\text{NH}_3\text{-N}$ at a higher temperature. Similar to soluble COD, the extension of treatment time did not enhance the release of $\text{NH}_3\text{-N}$ to a significant level, which was consistent with previous studies [12, 35]. The increase of COD and $\text{NH}_3\text{-N}$ should be attributed to the decomposition of intracellular proteins or exopolymers [36]. The solubilization con-

ditions may vary for different biomasses. In a previous study, Li *et al.* [4] found that a significant release of COD was achieved at 160°C for 60 min for the hydrothermal pretreatment of antibiotic mycelial residue. Further, increasing the temperature to 180°C resulted in a decrease in COD solubilization, which was attributed to the polymerization of smaller molecules. Based on the above results, hydrothermal treatment is found feasible for quickly removing antibiotics and enhancing the solubility of the OTC fermentation residue, and the hydrolyzed residue might be a potential substrate for further use.

3.3. Changes of *tet* genes after Hydrothermal Pretreatment

The quantifications of three *tet* genes namely *tetA*, *tetO* and *tetX* were conducted to examine their response to different hydrothermal treatments with a fixed retention time of 5 min. The occurrence of the *tet* genes is presented in copy numbers per dry mass (Fig. 2a) and normalized to 16S rRNA genes (Fig. 2 (b)), respectively. The absolute abundances of *tetA* and *tetX* were significantly ($p < 0.001$) decreased from 6.1×10^8 and 3.3×10^7 copies/g in the raw residue to 9.3×10^6 and 3.3×10^6 copies/g, respectively, at a temperature of 130°C. A similar reduction performance for both genes was achieved at 150 and 170°C treatments (Fig. 2 (a)). As for *tetO*, its abundance was reduced from 3.7×10^5 copies/g to 1.5×10^5 and 1.3×10^4 copies/g with the increase of the temperature to 150 and 170°C, respectively. Similar results on the reduction of the absolute abundances of the *tet* genes and some other resistant determinants by hydrothermal treatment of fermentation residues and waste activated sludge have been reported by previous studies [16, 37, 38]. This is understandable since abundant DNA including ARGs will be released during hydrothermal treatment, and it is known that DNA is sensitive to high temperatures, particularly at temperatures higher than 90°C [39]. As shown in Fig. 2 (b), however, the relative abundance of the three *tet* genes, particularly *tetA* and *tetX*, increased during the hydrothermal treatment. Wang *et al.* [37] also found that the relative abundances of *tet* genes in sewage sludge were increased after thermal hydrolysis treatment (120°C for 60 min), which suggested that the 16S rRNA genes are more susceptible to hydrothermal conditions than ARGs. It

Fig. 1. Solubilization effects of OTC residue under different hydrothermal treatment conditions: (a) Release of organic substances (sCOD), (b) Release of ammonia nitrogen. Error bars represent the standard deviation (SD).

Fig. 2. Change of *tet* genes in OTC fermentation residue after hydrothermal treatment at different temperature levels (a) Absolute abundance (b) Relative abundance. The values are mean + SD of duplicates.

should be noted here that the three *tet* genes showed different reduction during the hydrothermal treatment (Fig. 2 (a)). The reason, on the one hand, may be that they have different temperature sensitivity due to the difference in their gene sequence structures such as the GC content [40]. On the other hand, this variation might also be attributed to the different tolerance of their resistant bacteria hosts to high temperature. Further study is required to uncover the mechanism behind the different response of ARGs to the hydrothermal pretreatment.

3.4. Methane Formation Potential of the Hydrolyzed Residue

Fig. 3 shows the cumulative methane yields of different digesters containing raw residue (control) and treated residues over a period of 23 d. The cumulative methane yields were 73.7, 215.9, 656.8, and 439.0 NmL CH₄/gVS for the control, 130°C, 150°C, and 170°C treatments, respectively. Though the methane production profiles of the residues at 150°C and 170°C treatments did not reach the plateau, the above result already indicated that hydrothermal treatment at 150°C is the best choice among the tested three conditions for transforming the OTC fermentation residue into beneficial material for producing methane. The production of methane in the control was almost inhibited over the incubation period, which might be due to the high concentration of OTC in the raw residue inhibiting methanogenic metabolism. The inhibitory effect of anaerobic digestion process by OTC was reported previously by Beneragama et al. [41], who found that adding OTC at the dose of 30 - 90 mg/L in dairy manure resulted in 20.9% - 31.4% drop in methane yield. Recently, Tian et al. [26] found that OTC concentration higher than 4,180 mg/kg could severely inhibit methane production of mesophilic digestion using long-term chronic exposure experiment, due to the inhibition of fermenting and acidogenic bacteria. Besides removing the inhibitory effect of OTC, the hydrothermal pretreatment could also increase methane production as itself by hydrolyzing the OTC fermentation residue (Fig. 1 (a)). Interestingly, the methane yield of the OTC residue pretreated at 150°C was much higher than that at 130°C, though the soluble COD concentration was similar for both conditions (Fig. 1 (a)). The residue pretreated at 150°C showed a shorter lag phase compared

Fig. 3. Methane production of the raw and hydrolyzed OTC fermentation residues.

to other digesters and exhibited rapid methane formation. It is possible that the released biological macromolecules (e.g. proteins, complex sugars) were further transformed into smaller molecules such as monosaccharides and amino acids through further hydrolysis and degradation reactions [42, 43], which made them easier to be utilized for acetogenic and methanogenic metabolisms. Further increase of the temperature to 170°C, however, also led to a lower methane production yield. Carrère et al. [44] investigated the impact of hydrothermal pretreatment over a temperature range of 60 - 210°C on anaerobic digestion of waste activated sludge and found that methane production was enhanced up 190°C and further temperature increase adversely affected the process due to the formation of recalcitrant compounds, such as melanoidins and heterocyclic amines [45] depending on the condition of Maillard reaction and composition of reactants [46]. Dwyer et al. [47] also found more refractory COD was produced with the increase of temperature from 140 to 165°C during hydrothermal hydrolysis of activated

sludge. Another possible reason may be the adverse impact of the relatively higher ammonia concentration (976.6 mg/L) at the 170°C treatment, since the concentration of ammonia nitrogen higher than 800 mg/L could disturb the stability of anaerobic digestion [48, 49].

3.5. Changes of *tet* Genes after The BMP Test

The abundances of *tet* genes were detected before and after the BMP test to evaluate the impact of the hydrolyzed OTC residue on resistance development (Fig. 4). The total copy number of each *tet* gene in the mixtures of the substrate and inoculum before the BMP test was considered as a reference value for the evaluation. After digestion, *tetA* and *tetX* were significantly ($p < 0.01$) reduced under all treatments including the control system. *tetO* was enriched a little ($p < 0.05$) in the control, remained unchanged at 130°C, and decreased to below the detection level (2.9×10^2 copies/ μ L) at both 150 and 170°C treatments (Fig. 4). This result is in accordance with previous studies showing that mesophilic anaerobic digestion of sewage sludge could result in ARGs reduction even under antibiotic stresses [27, 37, 41, 50-52]. For example, Wang *et al.* [37] found that the ARGs removal efficiency of mesophilic digestion was approximately 50.8%. Zhang *et al.* [52] reported that *tetA* and *tetX* were significantly reduced when food waste was used as a substrate in mesophilic anaerobic digestion. It should be noted that short-term batch BMP test was used in this study with the main purpose to evaluate the methanogenic potential of treated residue, while long-term chronic exposure experiments should be performed to better check the induction of ARGs by the raw and thermo-hydrolyzed OTC residues.

Fig. 4. Absolute abundances of *tet* genes in the mixtures of OTC fermentation residues and inoculum before and after the BMP test.

3.6. Structure of Microbial Community after BMP Test

The microbial communities of sludge samples before and after the BMP test were analyzed using Miseq Sequencing. As shown in Fig. 5, the control sludge bacterial community was clustered together with the inoculum, while the three systems with hydrothermal pretreatment were clustered together. The control system did not have much anaerobic digestion activities due to the inhibition effect of OTC, which might be the reason why it showed

high similarity with the inoculum.

The sequences of bacterial communities were assigned to 15 phyla, with *Firmicutes*, *Synergistetes*, *Bacteroidetes*, and *Thermotogae* as the most predominant phyla in the four digesters (with relative abundance $> 5.5\%$ in all samples). As one of the most dominant phyla, *Firmicutes* occupied high proportions of 37.2%, 29.2%, 26.7%, and 7.2% in digesters of the control, 130°C, 150°C, and 170°C treatments, respectively. Members of *Firmicutes* phyla are important microorganisms involved in hydrolysis and fermentation during methanogenesis in anaerobic digestion [53, 54]. The other rare phyla include *Proteobacteria*, *Fibrobacteres*, *Spirochaetae* and *Atribacteria*. For the genus level, the most dominant genera were *norank_f_Synergistaceae*, *Mesotoga*, *Tepidimicrobium*, and *Proteiniphilum* (relative abundance $> 5.0\%$ in most of the samples) (Fig. 5). Members of *norank_f_Synergistaceae* within the phylum *Synergistetes* are syntroph essential for methane production [55], which were abundant in all the treatments. Genera including *Tepidimicrobium*, *Mobilitalea*, *Clostridium*, *Proteiniphilum*, *Caldicoprobacter*, *Bacteroides*, *Macellibacteroides*, *Ruminococcus*, and *Acetanaerobacterium*, all of which are common hydrolytic fermentative bacteria [56, 57], were enriched in the digesters fed with the hydrolyzed residues when compared with the control (Fig. 5). Their enrichment indicated an active methane metabolism in these digesters, which was consistent with the corresponding high methane productions revealed by the BMP test (Fig. 3). *Proteiniphilum* was especially enriched in the digested sludge of 150°C treatment with the relative abundance reaching 6.4% compared to 0.8%, 2.6%, 4.9% and 2.5% in the inoculum and samples of the control, 130°C and 170°C treatments, respectively (Fig. 5). *Proteiniphilum* belonging to family *Porphyromonadaceae* of phylum *Bacteroidetes* is known as amino acids degrader [58] and propionate-producing bacteria [59]. Zhang *et al.* [60] found that the presence of *Proteiniphilum* in anaerobic digestion was important for the optimization of volatile fatty acids composition and acceleration of methane production.

Based on our recent study [26], OTC mainly inhibits the fermenting and acidogenic bacteria of the methanogenic communities during mesophilic digestion, while methanogens remain active even under the OTC dose of 1,000 mg/L. So, in this study, the primers set of 515F/907R, which mainly targets the bacterial 16S rRNA gene [26, 28], was used to follow the different response of microbial communities. However, considering the importance of methanogenic archaea for process stability and the primers set could also amplify archaeal 16S rRNA gene sequences. As shown in Table S3, the sequences belonging to archaea accounted for a very low ratio ($< 5.5\%$) in the total sequence data in all the samples, which was in line with expectation considering the specificity of the primers. The result also indicated that the archaeal abundance is quite lower than the bacterial abundance, which was also consistent with previous studies [26]. As for the composition of the archaeal communities, *Methanobacterium* and *Methanolinea* were the dominant hydrogenotrophic methanogens, and *Methanosaeta* was the dominant aceticlastic methanogen in the inoculum and digested sludge samples after BMP test (Fig. S1 and Table S3). It suggested that further studies that use specific primers for detecting bacteria and archaea separately and are conducted for a longer anaerobic digestion time are required to have a better understanding of the microbial community features.

Fig. 5. Composition of bacterial communities at the genus level after the BMP test.

4. Conclusions

The present study investigated the effects of hydrothermal pretreatment on the removal of antibiotics from the OTC fermentation residue. Hydrothermal pretreatment at various temperatures of 130–170°C for 5 min efficiently removed the antibiotics (> 99.9%), decreased the abundance of the *tet* genes, and increased the solubilization of organic matter in the substrate. Further use of the hydrolyzed substrate for methane production revealed that pretreatment at 150°C for 5 min resulted in significantly higher methane production and a further decrease in *tet* genes. Increasing pretreatment temperature to 170°C resulted in lower performance in methane production, probably caused by the formation of recalcitrant compounds and inhibition of ammonia. This study provides the basis for a feasible treatment option for OTC fermentation residue through the combination of anaerobic digestion with the hydrothermal pretreatment.

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Author Contributions

M.A. (PhD) carried out the experiments, collected and analyzed the data and wrote the manuscript. Z.T. (PhD) conceived of the

presented idea, carried out the experiments, and wrote the manuscript. Y.Z. (Professor) supervised the project, revised and approved the manuscript to be published. Y.M. (Professor) supervised the project, revised and approved the manuscript to be published. W.Y. (PhD) commented and contributed to the final manuscript. L.D. (PhD) commented and contributed to the final manuscript.

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