Pretreatment of spiramycin fermentation residue using hyperthermophilic digestion: quick startup and performance
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ABSTRACT
This study aimed to evaluate the feasibility of hyperthermophilic anaerobic digestion at 70 °C in the pretreatment of spiramycin fermentation residue. By feeding municipal excess sludge under a solid retention time of 5 days, the hyperthermophilic digester was successfully started up within 3 days from mesophilic digestion by a one-step temperature increase from 35 to 70 °C. MiSeq sequencing showed the fast establishment of thermophilic fermenting bacterial communities in 3 days immediately after the temperature increase, with increases in abundance of Coprothermobacter, Spirochaetaceae_uncultured and Fervidobacterium from <0.001%, 1.06% and <0.001% to 33.77%, 11.65% and 3.42%, respectively. The feasibility of hyperthermophilic digestion for spiramycin residue was evaluated in batch experiments for 7 days. Hyperthermophilic digestion considerably reduced antibiotic concentrations, with removal efficiencies of 55.3% and 99.0% for the spiramycin residue alone and its mixture with hyperthermophilic sludge, respectively. At the same time, the abundances of four macrolide–lincosamide–streptogramin resistance genes were also reduced within 7 days, due to the decrease of their corresponding hosts. These results suggest that hyperthermophilic digestion could easily be started up from mesophilic digestion and might be a suitable pretreatment approach for spiramycin residue.

Key words | antibiotic resistance genes, hyperthermophilic digestion, mesophilic anaerobic digestion, spiramycin fermentation residue, startup

INTRODUCTION
The resistance of bacteria to antibiotics has become a global concern. Along with hospital and agricultural sources, wastes generated from antibiotic production processes are a major pathway for releasing antibiotics into the environment (Liu et al. 2012). China is the largest producer of bulk antibiotics in the world (Zhu et al. 2013). Every year millions of tons of antibiotic mycelial residues are generated from their fermentation production processes, which are listed as hazardous materials (MEP of PRC 2008) as they usually contain extremely high concentrations of residual antibiotics (Liu et al. 2012). At present, incineration is the most common method of disposing of such biomass wastes, but it requires expensive combustion equipment and carries a high treatment cost (Li et al. 2015). Cost-effectively disposing of antibiotic-containing residues has become an urgent requirement for manufacturers of antibiotics.

Anaerobic digestion as a widely used technology for the treatment of biosolids has been demonstrated to be effective in the reduction of antibiotic resistance genes (ARGs) (Sun et al. 2016; Tian et al. 2016). However, the application of conventional anaerobic digestion in the treatment of antibiotic production residues is often challenged by the presence of high concentrations of residual antibiotics, resulting in the inhibition of methane production and development of antibiotic resistance (Tian et al. 2018). Hyperthermophilic digestion operated at 70 °C, on the other hand, has recently been developed as an effective pretreatment technology for biosolids to improve their anaerobic digestion performance (Wu et al. 2016). Coupling hyperthermophilic and conventional digestion has been reported to achieve higher solids removal and net energy yield (Nazari et al. 2017). Since the hydrolysis of many antibiotics and the elimination of
various ARGs could be promoted at elevated temperatures (Diehl & LaPara 2010; Yi et al. 2017a), it is thus speculated that hyperthermophilic digestion may provide another advantage in treating antibiotic production residues by removing residual antibiotics and controlling ARG development. However, the feasibility of the hyperthermophilic process for antibiotic production residues treatment remains unclear.

This study aimed to explore the feasibility of applying hyperthermophilic digestion to the pretreatment of spiramycin production residue. Hyperthermophilic digestion was first started up from a mesophilic digester using the strategy of one-step temperature increase from 35 to 70 °C. The bacterial community succession and the changes in the abundance of four macrolide–lincosamide–streptogramin (MLS) resistance genes in the digested sludge were followed during the transition period by Illumina MiSeq sequencing and quantitative PCR, respectively. Then, the degradation of spiramycin and the development of antibiotic resistance during hyperthermophilic digestion of the fermentation residue were evaluated using bench experiment. The results of this study will provide scientific guidance for the safe treatment of antibiotic production residues.

MATERIALS AND METHODS

Startup and operation of the hyperthermophilic digester

A bench-scale anaerobic reactor was set up using a stirred glass tank with a working volume of 2 L. The temperature was controlled at 35 or 70 °C by circulating hot water in the water jacket. The reactor was initially inoculated with mesophilic digested sludge (35 °C) from Gaobeidian Wastewater Treatment Plant in Beijing, from which sewage sludge, a mixture of primary sludge and secondary sludge fed to the reactor, was also collected. The characteristics of the substrate are listed in Table S1 (available with the online version of this paper). The reactor was maintained at mesophilic conditions (35 °C) for more than 100 days with a solid retention time (SRT) of 20 days and was at steady state before the experiment started. Characteristics obtained from the steady state mesophilic operation were used as initial values. On day 1, the temperature of the reactor was directly increased to 70 °C, and each day after that, 400 mL mixture was withdrawn from the reactor, and replaced with the same volume of feed sludge to achieve the SRT of 5 days. Twenty days after startup, hyperthermophilic sludge from the reactor was supplied as seed sludge for the batch tests.

Total suspended solids (TSS) and volatile suspended solids (VSS) were analyzed according to Standard Methods (APHA 2012). The pH was measured simultaneously at the time of sampling with a portable meter (SevenGo Duo pro SG78, Mettler Toledo, Switzerland). Soluble chemical oxygen demand (sCOD) was determined after centrifugation at 10,000 rpm for 10 min and passing the supernatant through 0.22 μm filter. Volatile fatty acids (VFAs) were quantified using gas chromatography (Shimazu GC2010-plus, Japan) with a flame ionization detector and a capillary column (Restek Stabilwax-DA, 30 m × 0.35 mm × 0.1 μm) according to Tian et al. (2015).

Batch tests

To assess the feasibility of hyperthermophilic digestion for spiramycin production residue, fermentation residue was collected from an antibiotic manufacturing plant in Wuxi city, Jiangsu Province, China. The batch tests were carried out in duplicate in 500 mL serum bottles containing 400 mL of either spiramycin fermentation residue alone or hyperthermophilic sludge mixture with spiramycin fermentation residue at 1:1 ratio. The serum bottles were sealed with covers supplemented with an automatic mixer and incubated in water bath at 70 °C for 7 days. The samples were taken on days 0, 1, 3, 5 and 7. The effectiveness of the hyperthermophilic digestion process for treatment of spiramycin fermentation residue was evaluated based on the solubilization degree (sCOD and VFAs), reduction of spiramycin concentration and evolution of ARGs. All measurements were conducted in triplicate and expressed as means ± standard deviation.

DNA extraction

DNA was extracted with a FastDNA SPIN kit for soil according to the manufacturer’s instructions. The DNA concentration was determined with a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, USA).

Quantitative PCR analysis

For each sample, bacterial 16S rRNA gene and four MLS resistance genes, namely *ermF*, *ereA*, *mefA* and *ermX*, were quantified by SYBR green quantitative real-time PCR using LightCycler 96 (Roche Diagnostics GmbH, Germany) as
previously described (Tian et al. 2016). The amplification was carried out in a 25 μL reaction mixture. All reactions were performed in triplicate. The thermal program was set as one cycle of 95 °C for 10 min followed by 40 cycles of 95 °C for 15 seconds 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 (available online). Gene expression was calculated using software supplied with LightCycler. A standard curve was generated using known concentrations of standard plasmids containing each target gene. The correlation coefficients (R²) for standard curves and amplification efficiencies [E = (10⁻¹/slope – 1) × 100%] based on curve slopes were calculated to ensure reliable amplification. The R² values for all curves were over 0.99 and efficiency (E) between 92% and 108%. The specificity of the PCR was also assured by melting curves and gel electrophoresis.

Quantification of spiramycin

Ten milliliters of extraction solution (acetonitrile : water 1:1 (v/v)) was added to the freeze-dried sample. The samples were vortexed for 30 seconds, sonicated for 20 min and centrifuged at 3,210 g × 10 min. The supernatant was transferred to 500 mL bottles. These steps were repeated three times for each sample. A volume of 500 mL ultra-pure water was added to each sample and then alkalized by NaOH (0.1 M) to pH 11. Samples were extracted using Oasis HLB extraction cartridges (Waters, USA). The cartridges were conditioned by elution with 5 mL methanol followed by 5 mL ultrapure water. An aliquot of sample (330 mL) was passed through an HLB cartridge with a flow rate of 1 drop/s. The cartridge was then washed with 3 mL ultrapure water. The antibiotic fraction was eluted with 6 mL methanol containing 4% ammonia water (v/v). The obtained extracts were concentrated to near dryness under a gentle stream of nitrogen and then dissolved in 1 mL mixed solution composed of 0.1 M ammonium acetate containing 15% acetonitrile. The extract was again filtered through 0.22 μm and the concentration of antibiotic was analyzed using high performance liquid chromatography (HPLC-MS/MS) according to Montesdeoca-Esponda et al. (2012).

16S rRNA gene amplification, sequencing and data processing

An aliquot (50 ng) of purified DNA from each sample were used as a template for amplification of 16S rRNA genes. The V4-V5 hypervariable regions of bacterial 16S rRNA genes were amplified using the primers 515F and 907R (Xiong et al. 2012). Barcodes unique to each sample were incorporated before the forward primers, which allowed the identification of each sample in a mixture for an Illumina sequencing run. DNA was amplified in triplicate for each sample following a protocol described previously. PCR amplicons were pooled together and purified by agarose gel DNA purification kit (TaKaRa). The purified amplicons from different samples were then mixed to achieve equal mass concentrations in the final mixture, which were sent out to Majorbio Co. Ltd in Beijing for small-fragment library construction and pair-end sequencing using the Illumina MiSeq sequencing system (Illumina, USA).

Sequencing reads were assigned to each sample according to the unique 6-bp barcode for each sample. Pairs of reads from the original DNA fragments were merged using FLASH (Magoc & Salzberg 2011), and then were filtered using QIIME quality filters. PCR chimeras were filtered out using UCHIME (Edgar et al. 2011). The taxonomic classification of the sequences was carried out using the Ribosomal Database Project (RDP) Classifier at the bootstrap cutoff of 80%. The raw reads obtained from this study were deposited in the NCBI Sequence Read Archive under accession no. SAMN07375101 to SAMN07375109.

Statistical analysis

The generation of plots for the change of ARGs, microbial taxa, and spiramycin was performed with Origin 8.0 (OriginLab, USA). Hierarchical cluster analysis was conducted using Paleontological Statistics software (PAST 3.07). Pair-Sample t-tests were conducted using Origin 8.0 software to determine whether quantitative PCR-based ARG abundances were significantly different between samples. A p-value of <0.05 was considered to indicate significance. Network analysis was performed to visualize the correlations among ARGs and bacterial taxa based on the random matrix theory-based network inference method across all sludge samples from the transition period by using the online analysis pipeline at http://ieg2.ou.edu/MENA. Network visualization was conducted on the interactive platform of Gephi (version 0.9.1).

RESULTS AND DISCUSSION

Quick startup of the hyperthermophilic digestion

As shown in Figure 1, the concentrations of sCOD and VFAs reached a stable state 3 days after startup. The initial
Previous studies on hyperthermophilic digestion mainly focused on its improved solubilization efficiency for sewage sludge or food waste under stable conditions (Wu et al. 2016; Nazari et al. 2017), but little information is available for its startup, which is important for the practical application of the hyperthermophilic process. In this study, the above performance results demonstrate that hyperthermophilic anaerobic digestion could be rapidly started up from mesophilic digestion using the one-step temperature increase strategy, which has been successful for the fast startup of thermophilic anaerobic digestion (Tian et al. 2015).

The succession of bacterial communities during the startup of the hyperthermophilic process

sCOD of the digested sludge was 703.5 mg/L (day 0). It increased drastically in the first 3 days, and then remained stable up to the end of the experiment with values ranging from 4,680–5,895 mg/L (Figure 1). Similar to a previous study on excess activated sludge (Ariunbaatar et al. 2015), hyperthermophilic anaerobic digestion could lead to significant degradation of microbial cells and the release of soluble COD. A similar trend could be observed for the VFA. The concentration of acetate, and propionic, butyric, isobutyric, valeric and isovaleric acids increased from 264 ± 57, 96 ± 7, 22 ± 2, 16 ± 5, 45 ± 4 and 6 ± 3 on day 0 to 2551 ± 198, 495 ± 38, 71 ± 9, 154 ± 14, 31 ± 10 and 275 ± 27 mg/L, respectively, after 5 days (Figure 1). Both sCOD and VFAs rapidly increased immediately after the temperature increase, indicating that digested sludge maintained a high hydrolyt-fermentative activity after a rapid increase in temperature. Meanwhile, accumulation of VFAs in the digester is an indicator of inefficient conversion of organics into methane, which matched the result that no methane production was observed throughout the experiment, indicating the inhibition of the methanogenic activity after the temperature increase. Riau et al. (2010) also found that increasing the temperature to 70 °C suppressed methane production and increased the accumulation of VFAs. In spite of the accumulation of VFAs, however, the pH of the digested sludge remained stable at 7.2 ± 0.4 over the experiment, which was also observed by Bolzonella et al. (2007).

The TSS and VSS contents of the sludge fluctuated between 25–26 g/L and 15–16 g/L respectively with their removal rates at 6.11% and 6.51%, respectively, throughout the whole experiment. Previous studies on hyperthermophilic digestion mainly focused on its improved solubilization efficiency for sewage sludge or food waste under stable conditions (Wu et al. 2016; Nazari et al. 2017), but little information is available for its startup, which is important for the practical application of the hyperthermophilic process. In this study, the above performance results demonstrate that hyperthermophilic anaerobic digestion could be rapidly started up from mesophilic digestion using the one-step temperature increase strategy, which has been successful for the fast startup of thermophilic anaerobic digestion (Tian et al. 2015).

The succession of bacterial communities during the startup of the hyperthermophilic process

As shown in Figure 2(b), Firmicutes, Proteobacteria, Spirochaetae, Bacteroidetes, Actinobacteria, and Chloroflexi were the dominant phyla and accounted for more than 80% of the total microbial community in sludge samples. Firmicutes was the most dominant phylum, members of which were common anaerobic fermenting bacteria (Zhou et al. 2016), whose abundance gradually increased from 17.7% on day 0 to 47.9% on day 20 (Figure 2(b)). Spirochaetae also showed an increasing trend, whose abundance increased significantly from 1.1% to 11.3% in the first 3 days after the temperature increase. Conversely, the abundances of Chloroflexi and Bacteroidetes decreased from 19.5% and 14.1% to 3.1% and 4.5%, respectively, during the startup period. The abundances of Proteobacteria and Actinobacteria varied between 14.5–22.1% and 6.4–14.6%, respectively, throughout the startup period.

To further infer the functional adaption of the bacterial community, 10 bacterial genera exhibiting significant increasing or decreasing trends were picked up, as shown in Figure 2(c) and 2(d). Two potential thermophilic genera, Coprothermobacter and Spirochaetaeae_uncultured showed significant enrichment after the temperature increase.
The abundance of *Coprothermobacter* increased from <0.001% on day 0 to 33.77% on day 20, which was the most abundant genus in the hyperthermophilic sludge. The dominance of *Coprothermobacter* could be associated with the complexity of the organic substrate and the solubilization of proteins into small molecules (Pervin et al. 2013). *Spirochaetaceae_uncultured* as the second largest group in the hyperthermophilic sludge increased in abundance from 1.1% on day 0 to 11.1% on day 3, and then kept a relatively high level (12%–15.9%). Spirochaetal communities are assumed to be glucose fermenters and have been reported to metabolize acetate more actively (Lee et al. 2013). Therefore, the rapid increase of *Spirochaetaceae_uncultured* in the first 3 days could possibly be correlated to the drastic increase of acetate production (Figure 1). *Fervidobacterium*, *Rhodobacter*, *Lactivibrio*, and Geodermatophilaceae_uncultured also showed increased trends (Figure 2(c)). *Fervidobacterium* as a thermophilic obligatory anaerobic fermenting bacteria (Podosokorskaya et al. 2011), was previously found to be the most dominant fermenting bacteria (28.7%) in thermophilic anaerobic digestion (55 °C) (Tian et al. 2015). Its lower abundance (3.4% on day 20) suggested that it might be less competitive than *Coprothermobacter* under a higher temperature (70 °C). The increase of the above thermophilic genera maintained the hydrolytic-fermentative activity of the digested sludge when mesophilic bacteria were inhibited after the temperature increase. The above results demonstrated that high temperature facilitated the proliferation of the thermophilic genera, particularly *Coprothermobacter* and *Spirochaetaceae_uncultured*, at the beginning of the transition period, which is the key for the fast startup of the hyperthermophilic digester.

At the same time, the abundances of some potential mesophilic genera decreased (Figure 2(d)). At the initial stage on day 0, unclassified Bacteroidetes, Anaerolineaceae_uncultured, Sedimentibacter, and Planctomycetes_uncultured were dominant with their relative abundances at 5.3%, 4.7%,
3.4% and 2.0%, respectively. After the increase of temperature, these bacterial genera lost their dominance and only accounted for 2.2%, 0.8%, 0.01% and 0.5%, respectively, on day 20.

Changes of MLS ARGs during the transition period

As shown in Figure 3, four MLS ARGs, ereA, ermF, ermX and mefA, were successfully detected, with ereA and ermF being most abundant; their abundances in the substrate were reduced after digestion treatment. The total abundance of the four MLS genes in hyperthermophilic sludge on day 20 was $1.64 \times 10^7$ copies/mL, which was much lower ($p < 0.05$) than in the inoculated mesophilic sludge ($2.71 \times 10^7$ copies/mL) and the substrate ($5.77 \times 10^7$ copies/mL) (Figure 3). The effect of temperature on the reduction of ARGs through anaerobic digestion has already been well investigated. Diehl & LaPara (2010) examined the removal of ARGs from untreated wastewater solids at the bench scale and confirmed that removal efficiency of ARGs increased as a function of temperature. Sun et al. (2016) also reported that thermophilic anaerobic digestion reduced the abundance of mesophilic bacteria carrying ARGs. Considering the significant change in bacterial community composition in the digested sludge during the transition period, bacterial community shift might be one of the drivers impacting ARGs profiles. Network analysis, which has been reported as a reasonable tool for predicting the possible host of ARG in complex environments, including anaerobic digested sludge (Tian et al. 2016), was further used to correlate bacterial taxa with four MLS genes. Figure 4 illustrates the networks among ARGs and bacterial taxa. ereA, ermX, mefA, and ermF were possibly hosted by 62, 42, 18 and 3 bacterial taxa, respectively. Many of the coupling relationships, such as Paludibacter and Arenimonas carrying ereA, and Lysinibacillus and Ruminiclostridium carrying ermX, have been verified in previous studies (Table S3, available with the online version of this paper). The potential host information of the targeted ARGs will be helpful in understanding the controlling mechanism of ARGs during hyperthermophilic digestion.

Hyperthermophilic digestion of spiramycin fermentation residue

Change of solubility

Batch experiments were carried out over a period of 7 days to evaluate the performance of hyperthermophilic digestion with and without inoculation of hyperthermophilic sludge on the treatment of spiramycin fermentation residue. As shown in Figure 5(a), the concentration of the total VFAs for the inoculated system increased from 4,818 mg/L on day 0 to 8,912 mg/L on day 7, while the control system only varied from 5,222 mg/L to 6,826 mg/L. This result indicated that hydrolytic-fermenting bacteria in the hyperthermophilic microbial community promoted the solubilization of spiramycin fermentation residue.

Degradation of spiramycin

The residual concentration of spiramycin in the real fermentation residue was around 240 mg/L, which, to our knowledge, is the highest concentration ever reported in environmental samples. As shown in Figure 5(b), the residual concentration of spiramycin in the hyperthermophilic sludge inoculated system decreased from 119.3 ± 0.50 mg/L to 1.2 ± 0.3 mg/L in 7 days, while in the control system it decreased from 240.9 ± 9.12 mg/L to 107.7 ± 9.11 mg/L. Inoculation of hyperthermophilic sludge to the treatment system had diluted the residual concentration of spiramycin. It was clear that hydrolysis of spiramycin might have occurred during hyperthermophilic digestion, and inoculation of the hyperthermophilic sludge promoted the hydrolysis process. Yao et al. (2017) also found spiramycin was not degraded under anaerobic conditions until waste activated sludge or mesophilic digested sludge was added. In addition to hydrolysis, among the four MLS genes detected in the hyperthermophilic sludge, ereA could encode an esterase, which can inactivate erythromycin by lactone ring cleavage (Ounissi & Courvalin 1985). Considering the similar molecular structure of erythromycin and spiramycin, the host of the ereA gene in hyperthermophilic sludge might also contribute to the biodegradation of spiramycin. However, further

Figure 3 Absolute abundance of four ARGs detected during the startup period of the hyperthermophilic process. Bars represent standard errors.
studies are required to confirm the biodegradation of spiramycin during hyperthermophilic digestion.

Change of MLS ARGs

Because none of the MLS genes were detected in the residue (Figure 5(c)), the four MLS genes (ereA, ermF, ermX and mefA) detected in the hyperthermophilic sludge were chosen to examine the performance of the hyperthermophilic process on controlling the development of ARGs during its treatment of spiramycin fermentation residue. As shown in Figure 5(c), the abundances of the four ARGs decreased significantly ($p < 0.05$) from $2.59 \times 10^6 \pm 2.38 \times 10^3$, $1.16 \times 10^5 \pm 5.90 \times 10^2$, $2.16 \times 10^4 \pm 0.10 \times 10^3$ and $3.37 \times 10^4 \pm 5.90 \times 10^3$ on day 0 to $4.29 \times 10^5 \pm 2.57 \times 10^3$, $1.90 \times 10^4 \pm 1.40 \times 10^3$, $1.97 \times 10^3 \pm 0.50 \times 10^3$ and $2.06 \times 10^3 \pm 0.30 \times 10^3$ on day 7, respectively, during the batch experiment. The total abundance of the four ARGs decreased by 83.6% in spite of the presence of spiramycin, showing that hyperthermophilic digestion favored the control of the MLS genes. ARGs change mainly through the horizontal and vertical pathways (Tian et al. 2014). Our previous study has proved that the horizontal and vertical transfer-ability of ARGs decreased during anaerobic digestion when the temperature was increased from 35 to 55°C (Tian et al. 2015). Considering none of the MLS genes were detected in the residue (Figure 5(c)), the four genes in the
initial sludge in the batch experiment should originate from the added hyperthermophilic sludge and their potential bacterial hosts have already been revealed (Figure 4, Table S3). Therefore, to further explore the change mechanism of the four MLS genes, we firstly checked the contribution of the vertical pathway. As shown in Table S4 (available online), the abundances of \textit{ereA}, \textit{ermX}, and \textit{mefA} exhibited similar trends ($p < 0.05$) with their potential hosts during hyperthermophilic digestion, indicating that the blocking of the vertical transfer pathway might be an important mechanism for their reduction during the batch experiment. As for the \textit{ermF} gene, its decrease could be the result of the harsh environment restricting horizontal gene transfer (Tian et al. 2016). More research is required to verify the above hypothesis. On the other hand, considering high concentrations of antibiotics might negatively impact the anaerobic biological treatment of wastewater or biomass wastes (Wang et al. 2017; Yi et al. 2017b; Hu et al. 2018; Tian et al. 2018), the benefit of hyperthermophilic digestion for spiramycin residue should be further validated under higher spiramycin concentrations.

**CONCLUSIONS**

In this study, hyperthermophilic digestion could be started up by quickly using the one-step temperature increase strategy from a mesophilic inoculum. Fast startup relied on the rapid establishment of the thermophilic fermenting bacterial community. Hyperthermophilic digestion showed a
promising application in the pretreatment of spiramycin fermentation residue by simultaneously achieving enhancement of solubility, reduction of spiramycin concentration and removal of ARGs. The above findings are useful for developing cost-effective processes for the safe disposal of antibiotic production residues. To further promote the full-scale application of the hyperthermophilic process in the treatment of antibiotic residues, the performance and operating cost of its combination with conventional anaerobic digestion, and the effects of spiramycin and other antibiotics on it should be investigated in the future.

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