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Rapid startup of thermophilic anaerobic digester to remove tetracycline and sulfonamides resistance genes from sewage sludge

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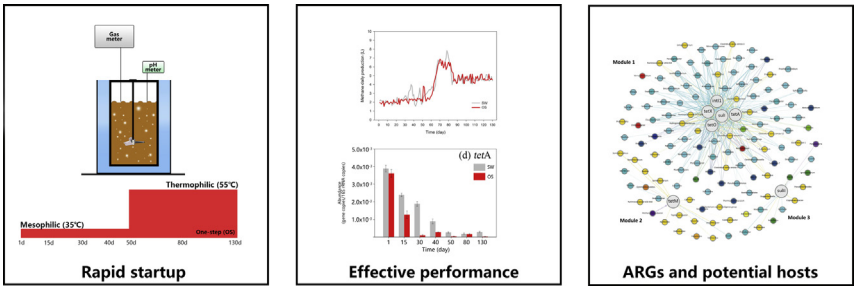
HIGHLIGHTS

- Rapid startup of thermophilic digester saved 20 days.
- Most antibiotic resistance genes were removed during thermophilic digestion.
- Network of antibiotic resistance genes and potential hosts were presented.

GRAPHICAL ABSTRACT



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ABSTRACT

Spread of antibiotic resistance genes (ARGs) originating from sewage sludge is highlighted as an eminent health threat. This study established a thermophilic anaerobic digester using one-step startup strategy to quickly remove tetracycline and sulfonamides resistance genes from sewage sludge. At least 20 days were saved in the startup period from mesophilic to thermophilic condition. Based on the results of 16S rDNA amplicons sequencing and predicted metagenomic method, the successful startup largely relied on the fast colonization of core thermophilic microbial population (e.g. *Firmicutes*, *Proteobacteria*, *Actinobacteria*). Microbial metabolic gene pathways for substrate degradation and methane production was also increased by one-step mode. In addition, real-time quantitative PCR approach revealed that most targeted tetracycline and sulfonamides resistance genes ARGs (*sul*I, *tet*A, *tet*O, *tet*X) were substantially removed during thermophilic digestion (removal efficiency > 80%). Network analysis showed that the elimination of ARGs was attributed to the decline of their horizontal (*int*I1 item) and vertical (potential hosts) transfer-related elements under high-temperature. This research demonstrated that rapid startup thermophilic anaerobic digestion of wastewater solids would be a suitable technology for reducing quantities of various ARGs.

1. Introduction

Bacteria becoming more resistant to various antibiotics has been recognized in recent years (Pruden et al., 2013). Although many actions have been taken to reduce the abuse of antibiotics, a continual increase in bacterial resistance to antibiotics was observed (Diehl and Lapara,

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2010). One reason is that the resistance can be conferred among environmental bacteria through the transfer of antibiotic resistance genes (ARGs). Such bacteria can be considered as potential hosts to transfer resistance to pathogenic bacteria (Wright, 2007). Proliferation of ARGs among bacteria, such as *Enterobacteriaceae* (a broad-spectrum β-lactamase producing bacteria) and *Enterococcus* (a vancomycin-resistant bacteria), resulted in the increasing morbidity and mortality of infections from multiantibiotic-resistant pathogens (Wright, 2010).

Municipal sewage collected by wastewater treatment plants (WWTPs) is a pertinent reservoir for ARGs (Lapara et al., 2011; Yuan et al., 2016). Because sewage contained substantial quantities of antibiotics, antibiotic resistance bacteria (ARBs) and ARGs originating from human gastrointestinal tract, livestock and poultry farm or medical industry (Zhang et al., 2009). During wastewater treatment, some ARGs might proliferate in sewage sludge due to the preferential survival and selection pressure of ARBs (Munck et al., 2015). Several typical ARGs (e.g. tetracycline resistance genes and sulfonamides resistance genes) have been widely detected from worldwide WWTPs (Bing et al., 2015; Hou et al., 2016; Jiang et al., 2017; Zhang and Zhang, 2011). What's worse, ARGs originating from sewage sludge escaped from WWTPs facilities to soil system due to their land application of residual solids, such as a fertilizer or a soil conditioner (Ghosh et al., 2009a; Munir et al., 2011). Thus, WWTPs are important nodes to help slowing the spread of ARGs into downstream natural environment (Pruden et al., 2013; Zhang et al., 2015).

Anaerobic digestion (AD) has been widely used to dispose sewage sludge at WWTPs, due to the reduction of sludge volume, production of renewable energy (methane) and removal of pathogens (Gou et al., 2014; Xu et al., 2017). Nowadays, AD is also likely to reduce the persistent ARGs by some degree. However, conclusions are not universal in previous studies (Diehl and Lapara, 2010; Ghosh et al., 2009a; Ma et al., 2011a; Tian et al., 2015; Tian et al., 2016; Zhang et al., 2015). Some research reported ARGs were considerably removed during AD process. For example, Zhang et al. (2015) found that 8 out of 35 ARG types (including tetracycline resistance genes) could be removed >90%. Diehl et al. also suggested the ability of full-scale anaerobic digesters in removing tetracycline resistance genes (*tet*A, *tet*L, *tet*O, *tet*W, and *tet*X) (Diehl and Lapara, 2010). However, other studies indicated that some ARGs types were slightly reduced or even rebounded using anaerobic digesters (Ma et al., 2011b; Zhang et al., 2015). This may be attributed to the complex interactions among ARGs, microbes, digestion conditions, and related-metabolic pathways involved in AD process, which are still considered as a "black box". Horizontal gene transfer (by the transfer of mobile element carrying ARGs) and vertical gene transfer (by the proliferation of ARGs' hosts) between different bacterial cells were recognized as the main spread ways of ARGs (Sørensen et al., 2005). Therefore, studying the fate of ARGs and their horizontal/vertical transfer-related elements simultaneously can provide comprehensive insights into controlling mechanism of ARGs during AD process.

Temperature is one of critical parameters determining the stability and performance of AD. Studies suggested that high-temperature digestion is also able to inactivate ARB and ARGs in sludge (Diehl and Lapara, 2010; Ghosh et al., 2009a; Ma et al., 2011b). AD technology is usually conducted at mesophilic (ca. 35 °C) or thermophilic (ca. 55 °C) condition to supply an optimum parameter for the growth of different microbes (Diehl and Lapara, 2010). Prior studies indicated that thermophilic digestion generally outperforming mesophilic digestion in the reduction of ARGs (Diehl and Lapara, 2010; Tian et al., 2015; Tian et al., 2016). However, most of current full-scale digesters adopted mesophilic digestion. Expect for a higher operation cost, another main obstacle for the application of thermophilic digestion is the longer startup time (Tian et al., 2016). Since lacking of existing thermophilic seed sludge, it is usually required to domesticate from mesophilic to thermophilic condition in a full-scale digester, which was supposed to be time-consuming (Tian et al., 2015). A stable AD system highly depends on the complex interaction among different microorganisms participated in hydrolysis, acidogenesis, acetogenesis and methanogenesis processes (Rivière et al., 2009). Key to successful transformation depends on the formation of a mature thermophilic community for AD operation. But it is reported that abrupt temperature-raising will disturb original mesophilic population (e.g. methanogens) during domestication process, making the AD system unstable (Griffin et al., 1998).

To maintain the reactor's stability and save startup time, a rapid startup mode of thermophilic anaerobic digester is proposed in this study. Digestion performance is continuously monitored to compare the influence of different temperature-raising strategies. The fate of ARGs, potential hosts and function gene pathways involved in AD process are jointly investigated by real-time quantitative PCR (RT-qPCR), high-throughput sequencing (HTS) and predicted metagenomic method. This study will be helpful to quickly establish thermophilic AD reactors and effectively control proliferation of ARGs.

2. Material and methods

2.1. Preparation of digestion substrate

SW and OS were initially inoculated with 2 L anaerobic seed sludge and 1 L feed sludge. Seed sludge was collected from a mature mesophilic anaerobic digester (Yang et al., 2016). Daily feed sludge (a mixture of secondary sludge and dewatered sludge) was sampled from Yuelu WWTPs, Changsha. Main characteristics were summarized in Table 1.

2.2. Startup of thermophilic digesters

Experiments were conducted in two continuous stirred-tank reactors with 3 L working volume (mixing speed is 60 rpm with a cycle of 1 min on and 10 min off throughout the experiment). Two reactors were operated at a constant hydraulic retention time (HRT) of 15 days, 200 mL of digested/untreated sludge was removed/refilled every day to maintain a constant working volume (Jang et al., 2016). Reactors' temperature was controlled by circulating hot water using heating rod. Initially, both reactors were operated at 35 °C (mesophilic condition) for 30 days until biogas production stabilized. Then, reactor SW (step-wise mode) increased digestion temperature from 35 °C to 55 °C gradually during 20 days with the raising interval of 1 °C/d. On day 50, reactor OS (one-step mode) increased temperature from 35 °C to 55 °C directly. Lastly, reactors SW and OS were operated at thermophilic condition (55 °C) over 80 days to reach a steady state (Table 2).

Table 1
Main characteristics of seed sludge and feed sludge used in this study.

Item	Seed	Feed
Volume (mL d^{-1})	–	200
pH	7.21 ± 0.1	6.82 ± 0.1
SCOD (g L^{-1})	0.74 ± 0.3	14.7 ± 2.8
TS (g L^{-1} substrate)	37.9 ± 0.2	479.5 ± 8.3
VS (g L^{-1} substrate)	9.3 ± 1.1	142.4 ± 4.8
VS/TS (%)	24.6 ± 0.7	29.7 ± 0.5
VFAs (mg L^{-1})	878.5 ± 36	1219.4 ± 81
*sul*I[a]	ND	(1.42 ± 0.2) × 10^{-2}
*sul*II[a]	ND	(3.37 ± 0.6) × 10^{-3}
*tet*A[a]	ND	(4.13 ± 0.7) × 10^{-3}
*tet*L[a]	ND	(3.27 ± 0.6) × 10^{-3}
*tet*M[a]	ND	(4.39 ± 0.2) × 10^{-3}
*tet*O[a]	ND	(1.78 ± 0.4) × 10^{-2}
*tet*W[a]	ND	(5.98 ± 1.1) × 10^{-4}
*tet*X[a]	ND	(3.37 ± 0.9) × 10^{-4}

[a] The relative abundance of ARGs was normalized by total 16S rDNA gene numbers.