Bioresource Technology 241 (2017) 1027–1035

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Sequential batch thermophilic solid-state anaerobic digestion of lignocellulosic biomass via recirculating digestate as inoculum – Part II: Microbial diversity and succession

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HIGHLIGHTS

- Effect of recirculating digestate as inoculum on microbial communities was studied.
- Microbes shifted toward stable state with increased diversity in the first 3 runs.
- Relative abundance of *Firmicutes* increased from 40% to 80% from run 1 to run 3.
- *Methanothermobacter* was enriched at volatile fatty acid (VFA) levels of 6–14 g/kg.
- Proportions of archaea rose from 1% to 5% from run 1 to run 4 when methane peaked.

ARTICLE INFO

Article history:
Received 26 April 2017
Received in revised form 2 June 2017
Accepted 3 June 2017
Available online 9 June 2017

Keywords:
Thermophilic
Solid-state anaerobic digestion
Microbiome
Illumina sequencing
16S rRNA gene

ABSTRACT

This study aimed to investigate the effect of recirculation of digestate as inoculum on the microbial communities in thermophilic solid-state anaerobic digestion (SS-AD) of yard trimmings. The SS-AD consisted of 4 consecutive runs (30 days/run), with digestate from the previous run being used as the inoculum of the subsequent run. Bacterial and archaeal communities (day 0, 4, 8, 12, 20, and 30) were examined using Illumina sequencing of 16S rRNA genes.

The results revealed substantial microbial succession toward increased diversity until run 3. The proportions of *Firmicutes* that contained cellulolytic bacteria doubled, which might explain the concomitantly increased cellulose degradation and volatile fatty acids (VFAs). *Clostridia* and *Thermotogae* appeared to correlate with VFAs. The VFA accumulation likely induced dynamic shifts of methanogens, particularly to hydrogenotrophic *Methanothermobacter*, implying that non-acetoclastic oxidative pathway dominated during the steady-state thermophilic SS-AD. This study suggested that recirculating SS-AD digestate might be an effective way for inoculation.

1. Introduction

Anaerobic digestion (AD) is a mature and common waste management technology that relies on microorganisms, both bacteria and archaea, to produce energy and reduce waste streams under oxygen-free conditions. Successful AD system performance primarily depends on the robustness of these microbes. Theoretically, the AD process involves four microbial metabolic steps: hydrolysis by hydrolytic bacteria that reduce substrate polymers to monomers; acidogenesis and acetogenesis by acid-forming bacteria that ferment monomers to volatile fatty acids (VFAs, primarily acetate), carbon dioxide (CO_2), and hydrogen (H_2) and other byproducts; and lastly, methanogenesis by methanogenic archaea that produce methane from the fermentation products, mainly acetate, CO_2, and H_2 (Li et al., 2011). Interactions among the diverse bacteria and archaea are complex and any imbalance may disturb or even cause the AD process to fail (Li et al., 2015).

Various AD systems and designs are available in terms of operating temperature, moisture, substrates, inoculation, and other

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http://dx.doi.org/10.1016/j.biortech.2017.06.011
0960-8524/

options. Thermophilic AD (55 °C) has been commercialized widely in Europe for treatment of animal manure and the organic fraction of municipal solid waste due to its enhanced degradation of organic matter and destruction of pathogens (De Baere and Mattheeuws, 2012). Increases in the generation of solid wastes, including municipal and agricultural wastes, have fostered the development of solid-state AD (SS-AD) that operates at >15% total solids (TS) content (Li et al., 2011). High TS content allows a small reactor volume, low energy input for heating, and prevention of floating or stratification of solids. In recent years, thermophilic SS-AD has attracted much attention due to its potential to convert lignocellulosic biomass to energy more efficiently than mesophilic SS-AD (Shi et al., 2013). In a recent study, a sequential batch thermophilic SS-AD of yard trimmings was developed that exhibited long-term stability when digestate was recirculated as the inoculum (Lin and Li, 2017). That study was conducted for 4 runs, with each run lasting 30 days. Interestingly, the system was found to gradually reach steady state by the 3rd run with slightly higher methane yields (up to 11.5%) and cellulose degradation (up to 55%) than in runs 1–2. The results suggest that recirculation of the SS-AD digestate as the inoculum might have provided microbes that had been acclimated to the substrates and operating conditions. Characterization of the diversity and successions of the microbial communities of this SS-AD system will increase understanding of the sequential batch thermophilic SS-AD process.

Numerous studies have analyzed the microbial communities in AD systems. Although early studies used community fingerprinting methods, such as denaturing gradient gel electrophoresis (DGGE), fluorescent in-situ hybridization (FISH), and restriction fragment length polymorphism (RFLP) (Cabezas et al., 2015; Hori et al., 2006; Shi et al., 2013), next-generation sequencing (NGS) technologies have proved to be much more powerful in providing detailed characterization of communities of both bacteria and archaea in anaerobic digesters (Li et al., 2015). Recent studies using NGS revealed a vast diversity of both bacteria and archaea in AD digesters fed different feedstocks and operated under different conditions (Guo et al., 2014; Li et al., 2015; Yi et al., 2014). However, few studies have investigated how recirculation of solid digestate as inoculum affects the diversity and succession of bacterial and archaeal populations in thermophilic SS-AD systems treating lignocellulosic biomass.

The objectives of the present study were to: (1) determine the bacterial and archaeal diversity and succession in a sequential batch thermophilic SS-AD operation that recirculated digestate as the inoculum; and (2) study the correlation among the microbial community dynamics, environmental factors, and reactor performance. This study on microbial communities of SS-AD with sequential batch operation may also provide some insights for commercial scale systems. Besides, understanding the microbiology of SS-AD is not only critical for understanding the process itself, but also useful for improving and optimizing the process.

2. Materials and methods

2.1. Sample information

The samples analyzed in the present study were collected in a previous study (Lin and Li, 2017). Briefly, a sequential batch thermophilic SS-AD system was developed by recirculating a portion of the digestate as the inoculum. Yard trimmings obtained from The Ohio State University's Wooster campus (Wooster, OH, USA) were used as the substrates. The dewatered effluent (CTRL1) taken from a commercial mesophilic liquid anaerobic digester (L-AD, TS < 15%) was used as the initial inoculum to prepare the thermophilic SS-AD digestate (CTRL2) for the subsequent sequential batch SS-AD operation. CTRL2 was obtained after incubation for 40 days. The sequential batch SS-AD operation included 4 runs with each run lasting 30 days. For each run, digestate from the previous run was used as the inoculum. Samples were collected at day 0, 4, 8, 12, 20, and 30 during each run and stored at −80 °C prior to microbial analysis. All the tests (including the preparation of CTRL2) were conducted in triplicate at 55 °C with a TS content of 22% and a substrate-to-inoculum (S/I) ratio of 1 (TS basis).

2.2. DNA extraction and 16S rRNA gene sequencing

Total metagenomic DNA was extracted from 0.25–0.3 g of each sample using the PowerSoil® DNA Isolation Kit (MOBIO Laboratories, Inc., Carlsbad, CA, USA). The DNA quality was verified using agarose gel (1.0%) electrophoresis, and the DNA concentrations were quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The DNA samples were submitted to the Molecular and Cellular Imaging Center (MCIC) on the Wooster campus for 16S rRNA amplicon library preparation and subsequent gene sequencing. Briefly, amplicon libraries were prepared by amplifying the V4–V5 hypervariable region of 16S rRNA gene with the primer set 515F and 806R, targeting both bacteria and archaea (Li et al., 2015). Then, the amplicon libraries were sequenced on an Illumina MiSeq using a 2 × 300 bp paired-end protocol.

2.3. Sequence data analysis

The Illumina sequencing data obtained from the MCIC were demultiplexed according to the dual indices attached to the sequences followed by trimming of those indices. The demultiplexed sequencing data were processed and analyzed using QIIME (v 1.9) (Caporaso et al., 2010) following the protocol developed by Nelson et al. (2014) with slight modifications. Specifically, the sequences corresponding to the forward and reverse primers were first trimmed from the demultiplexed reads using cutadapt (https://cutadapt.readthedocs.io/en/stable/index.html). The trimmed read pairs were merged into respective single sequences using SeqPrep (https://github.com/jstjohn/SeqPrep). The merged reads were quality filtered using a minimum Q score of 20 followed by a length-filtering step to obtain reads of 280–320 bp. The resulting sequences were assigned to species-equivalent operational taxonomic units (OTUs) at a 97% sequence similarity level using the open-reference OTU picking workflow, which included multiple steps of both reference-based OTU clustering (uclust_ref method against 2013-08 Greengenes database) and *de novo* OTU clustering (uclust method). Chimera checking was conducted on the aligned representative sequences of OTUs using ChimeraSlayer against the 2013-08 Greengenes. From the chimera-free OTUs, singleton OTUs were removed prior to further analysis. Alpha diversity metrics were calculated for each sample for Shannon and Simpson indices, phylogenetic distance, and observed number of OTUs. Beta diversity was calculated with the weighted UniFrac distance matrix and then visualized using principal coordinates analysis (PCoA).

The distribution of the 99 major bacterial genera (each represented by ≥0.005% of the total bacterial sequences) was further analyzed using the software generalized association plots (GAP) (Wu and Chen, 2012) as described elsewhere (Li et al., 2014). The abundance (represented by the number of sequences) of the genera was first log transformed for normalization and then visualized with a double hierarchical dendrogram, which was implemented in the GAP software (Chen, 2002; Wu et al., 2010). Furthermore, canonical correspondence analyses (CCA) were performed using the XLSTAT (version 2015) to examine correlations of bacterial and archaeal genera with environmental factors and reactor