Anaerobe 47 (2017) 8–17

Contents lists available at ScienceDirect

Anaerobe

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

Anaerobes in the microbiome

Effect of operating temperature on anaerobic digestion of the Brazilian waterweed *Egeria densa* and its microbial community

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ARTICLE INFO

Article history:
Received 9 December 2016
Received in revised form
14 March 2017
Accepted 15 March 2017
Available online 16 March 2017

Handling Editor: Andrew Bruce Onderdonk

Keywords:
Anaerobic digestion
Brazilian waterweed
Temperature
Microbial community

ABSTRACT

To develop an effective treatment for the globally invasive Brazilian waterweed *Egeria densa*, anaerobic digestion was observed at 37 °C, 55 °C, and 65 °C. The average methane production rate at 55 °C was 220 mL L^{-1} day^{-1}, which was two-fold that at 37 °C and 65 °C. Volatile fatty acid accumulation was detected under thermophilic conditions; however, although there was methane production, the system did not shutdown. The microbial communities differed between mesophilic (37 °C) and thermophilic (55 °C and 65 °C) conditions. A bacterial community consisting of the phyla Bacteroidetes (43%), Firmicutes (37%), Proteobacteria (9%), Synergistetes (5%), Spirochaetes (1%), and unclassified bacteria (5%) were detected under mesophilic condition. In contrast, the phylum Firmicutes was dominant under thermophilic conditions. In the archaeal community, *Methanosaeta concilii* (40%), *Methanolinea* sp. (17%), and unclassified euryarchaeota (43%) were detected under mesophilic condition. *Methanosarcina thermophila* (87% at 55 °C, 54% at 65 °C) and *Methanothermobacter thermautotrophicus* (13% at 55 °C, 46% at 65 °C) were detected under thermophilic conditions. At both 37 °C and 55 °C, acetoclastic methanogenesis likely occurred because of the lower abundance of hydrogenotrophic methanogens. At 65 °C, the growth of the acetoclastic methanogen *Methanosarcina thermophila* was limited by the high temperature, therefore, acetate oxidation and hydrogenotrophic methanogenesis may have occurred.

1. Introduction

The Brazilian waterweed *Egeria densa* is one of the most predominant invasive aquatic weed species, not only in Japan but also worldwide. It has a high growth rate and inhibits a wide range of aquatic systems [41,45,63]. The overgrowth of aquatic weeds, including *E. densa*, causes serious environmental and social problems, for example, changing of ecosystems, fishing interference, water stagnation, and strong odors released from putrid aquatic weeds [1,13,17]. In the southern basin (an area approximately 56 km^2) of Lake Biwa, which is the largest lake in Japan, aquatic weeds have propagated excessively, covering approximately 90% since 1994 [17]. In this area, excess aquatic weeds are harvested for lake management. The amount of harvested aquatic weeds reached >2600 tons (wet weight), and harvesting costs > USD 2.0 million every year [18]. Therefore, effective, low-cost treatment of aquatic weeds is required.

In recent years, anaerobic digestion (AD) has re-focused on wet organic waste treatment because it recovers biomethane and the nutrient-rich digestion fluid can be used for liquid fertilizer. In aquatic weeds, methane recovery is regulated by lignin content [27], which is one of the main components of the lignocellulosic biomass [56]. *E. densa* lignin content is 44–54 mg g-total $solids^{-1}$ [26,27,42], and methane yield is ~287–400 mL g-volatile $solids^{-1}$ by batch operation at 37 °C [26,27]. *E. densa* is a relatively degradable aquatic weed species and is a potential candidate for AD. Furthermore, to improve methane recovery from *E. densa*, various strategies, e.g., thermochemical pre-treatment [28], co-digestion with other biomass [68], and microbial electrolysis cells (MECs)-assisted AD [69], have also been attempted.

AD process is reacted with enzymatic activities which were produced by microorganisms. Operating temperature is an

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http://dx.doi.org/10.1016/j.anaerobe.2017.03.014
1075-9964/

important factor affecting microbial activity and methane production. The AD process is generally divided into four phases; hydrolysis, acidogenesis, acetogenesis, and methanogenesis [11]. Among these phases, hydrolysis is the most limiting step in the treatment of lignocellulosic biomass [15]. The hydrolysis phase is enhanced by increasing operating temperature [39,50,59,61]. AD treatment is usually carried out under either mesophilic (30–40 °C) or thermophilic (50–65 °C) conditions. Thermophilic AD treatment offers some advantages, such as higher metabolic and methane production rates, shortened retention time, and a greater degree of pathogen reduction, compared to mesophilic treatment [3,12]. However, thermophilic AD treatment also has some drawbacks, such as lower stability compared to mesophilic treatment, and the process shutdown happened by a pH drop due to the excessive volatile fatty acid (VFA) accumulation [21,57]. Excessive VFA accumulation is more likely to occur from easily degradable substrate such as food waste. However, because the lignocellulose degradation rate is relatively low [6,20], it is expected that the excess acid production is less likely to occur in food waste. In fact [15], and [32] reported that lignocellulose degradation was enhanced by thermophilic AD treatment. Therefore, a thermophilic operation may enhance methane production from aquatic weeds. Some archaeal species have an optimum growth temperature of approximately 65 °C (e.g., *Methanothermococcus* sp., *Methanothermobacter* sp., and *Methanobacterium* sp.) [62,66]. Zinder (1990) reported that the upper temperature limit for methanogenesis from acetic acid is 70 °C.

However, microbial diversity decreases with increasing AD temperature, which would negatively influence process performance [2,29]. High microbial diversity in the AD process is resistant against shock and pH fluctuations, some kinds of substrates lead to stable conditions [8]. Understanding of both process performance and microbial community during AD is necessary to determine the optimum operating conditions. The objectives of this study were to examine methane recovery for *E. densa* treatment under three different temperatures, 37 °C, 55 °C, and 65 °C, and to investigate the microbial community. The optimum operating conditions for the treatments are discussed in relation to microbial community.

2. Materials and methods

2.1. Reactor operation

The Brazilian waterweed *Egeria densa* was harvested from an artificial pond at Soka University, Hachioji, Tokyo, Japan. It was roughly shredded (approximately 0.5–1.5 cm) with a kitchen knife and stored at −20 °C until use. Before the experiment, the substrate was defrosted at room temperature. It was fed into a completely stirred tank reactor (CSTR) with an effective volume of 1.5 L once every 2 days at an organic loading rate (OLR) of 1.0 g-VS^{-1} L^{-1} day. The hydraulic retention time (HRT) was 30 days. For the AD inoculum, the mesophilic (37 °C) anaerobic sludge for treating domestic sewage sludge was obtained from Hokubu Sludge Treatment Centre in Yokohama (Kanagawa Prefecture, Japan). Before the experiment, it was degassed at 37 °C for 2 days to digest the residual organics. Trace elements were supplemented into the reactor with substrate [49]. The reactor was heated to 37 °C (days 0–30), 55 °C (days 35–66), and 65 °C (days 72–102) with a ribbon heater. No substrate was added prior to the temperature changes in order to remove the residue. The experiment was conducted in duplicate.

2.2. Physicochemical parameters

Total solids, volatile solids, pH, and soluble chemical oxygen demand (SCOD) were analyzed following methods in the Standard methods from APHA [5]. Lignocellulose (cellulose, hemicellulose and lignin) content was measured by detergent system [58] using fiber analyzer (Ankom, A-200, USA). Biogas (methane and carbon dioxide) composition was monitored with a gas chromatograph (SHIMADZU, GC-2014, Kyoto, Japan) equipped with a packed column (Shinwa Chemical Industries Ltd., Shincarbon ST, 6 m long, 3.0 mm i.d., Kyoto, Japan) and a thermal conductivity detector. The injector and detector temperatures were maintained at 120 °C and 260 °C, respectively. The column temperature was gradually increased from 40 °C to 250 °C. Helium was used as the carrier gas with a flow rate of 40 mL min^{-1}. Volatile fatty acid (VFA) composition (acetic, propionic, n-butyric, i-butyric, n-valeric, and n-valeric acid) was monitored with a gas chromatograph (Hitachi, G-3000, Tokyo, Japan) equipped with a capillary column (SHIMADZU GLC Ltd., Stabilwax, size × I.D. 30 m × 0.53 mm, df 1.00 μm, Tokyo, Japan) and a flame ionized detector (FID). The injector and detector temperatures were both maintained at 240 °C. The column temperature was gradually increased from 100 °C to 160 °C at 4 °C minutes^{-1}. Nitrogen was used as the carrier gas at a flow rate of 40 mL min^{-1}.

2.3. Community DNA extraction

Samples were obtained when methane production was stable at each temperature, and stored at −25 °C until the DNA extraction. These were examined for both bacterial and archaeal communities by the cultivation-independent method. Community DNA was extracted from about 5 g wet samples using an UltraClean™ Soil DNA Kit Mega Prep (Mo Bio Laboratories, CA, USA), according to the manufacturer's instructions.

2.4. 16S rRNA gene (16S rDNA) amplification

Community DNAs were used as the template DNA for polymerase chain reaction (PCR). Bacterial and archaeal 16S rRNA genes (16S rDNAs) were amplified by PCR using the universal oligonucleotide primers as follows: bacterial forward primer: B27F (5′-AGAGTTTGATCCTGGCTCAG), archaeal forward primer: A21F (5′-TTCCGGTTGATCCYGCCGGA) and universal reverse primer: U1492RM (5′-GGYTACCTTGTTACGACTT). Amplification by PCR comprised 30 cycles of 30 s at 94 °C, 30 s at either 61 °C (for bacteria) or 55 °C (for archaea), 2 min at 72 °C, and a final extension of 5 min at 72 °C [30] using *Ex Taq* DNA polymerase (Takara Bio, Shiga, Japan).

2.5. Clone library construction and analysis

The PCR products were purified using GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, USA), according to the manufacturer's instructions. Purified PCR fragments were cloned into the pT7 Blue T-vector (Novagen, Darmstadt, Germany) and the recombinant plasmids produced were transformed into *Escherichia coli* DH5 alpha cells. The transformants were plated on Luria-Bertani (LB) plates containing 100 μg mL^{-1} ampicillin (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 40 μg mL^{-1} X-gal (Takara Bio), and 0.5 mM IPTG (Takara Bio). Blue/white selection was conducted where individual white colonies were picked randomly and subcultured in 100 μl LB medium containing 100 μg mL^{-1} ampicillin using a 96-well plate at 37 °C overnight. The 16S rDNA inserted in the plasmid was amplified by PCR using 1 μl of the culture as the template with the primers T7P-F (5′-TAATACGACTCACTATAGGG) and T7U-R (5′-GTTTTCCCAGTCACGACGT) for bacteria, A21F and U1492RM for archaea. The PCR cycle for the primer pair T7P-F and T7U-R consisted of an initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for