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Enhancement of sludge granulation in anaerobic acetogenesis by addition of nitrate and microbial community analysis

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ABSTRACT

Sludge granulation is a key factor to sustain anaerobic systems operating efficiently and steadily. Nitrate as a H_2 consumer was added into anaerobic digesters to investigate its effects on the sludge granulation. The results showed that adding nitrate increased the sludge granule size by 289%, 325% and 790% with acetate, propionate and butyrate as substrates, respectively. Butyrate was preferable to the denitrifying bacteria because it was capable of releasing more electrons available for denitrification during acetogenesis. The analyses of fluorescence in situ hybridization, scanning electron microscope, and denaturing gradient gel electrophoresis indicated that denitrifying bacteria and volatile fatty acid (VFA)-oxidizing bacteria in the butyrate digester were richer than those in the other digesters. Taken together, addition of nitrate accelerated the decomposition of VFA and simultaneously improved the granulation of anaerobic process.

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1. Introduction

Anaerobic digestion is a widely-applied technology to decompose organics and simultaneously produce biogas from wastewaters and sludge [1]. Hydrogen partial pressure in the anaerobic system is required to be maintained at a quite low range to allow the anaerobic process continuously happening [2]. Hydrogen is produced from non-methanogenic microorganisms metabolizing the fermentation products (especially organic acids) and can be consumed by hydrogenotrophic methanogens with the reduction of CO_2 into CH_4 [3,4]. When the anaerobic system suffers from a load or acidic shock, hydrogenotrophic methanogens may be inhibited so as to decrease the hydrogen utilization [5]. It will cause the organic-acid decomposition to stop, leading to a high concentration of organic acid accumulation, further deteriorating the final methanogenesis [6,7].

Denitrification as an electron-accepting process is considered as an alternative to consume the hydrogen produced in anaerobic digestion [8]. If the hydrogen produced from volatile fatty acid (VFA)-oxidizing bacteria is consumed by denitrifying bacteria, both anaerobic digestion and denitrification is expected to be accelerated. Barber and Stuckey observed that denitrification accelerated the conversion of propionate and butyrate significantly and Lens et al. reported that addition of nitrate (2.3 gNO_3^-/g acetate) considerably improved the acetate removal efficiency of sulfidogenic reactors [8,9].

Granules have regular and dense structure with superior settling properties and high biomass retention, which is a key factor to withstand high-strength organic loads in anaerobic system and maintain its operation steady [10,11]. Anaerobic granular aggregate is composed of three layers [14,15]. In the outmost layer of the granules, hydrolytic and/or acidogenic bacteria are predominant, whereas the internal core of the granules prevailingly consists of methanogens. The syntrophic communities including hydrogen-producing bacteria and hydrogen-consuming bacteria are located in the middle layer. Recently, enhancement of granulation in anaerobic reactors by addition of nitrate has been reported [11–13,19,30,35]. Hendriksen and Ahring cultivated active denitrifying/methanogenic granules with big size and high metabolic activities [19]. Wang et al. and Green et al. also observed the enhanced granulation in a denitrification (upflow anaerobic sludge bed) UASB reactor [30,31]. In the presence of nitrate, the interspecies electron transfer between acetogenesis and denitrification is likely to improve the digestion rates and provide a protection for the inner methanogens, and then accelerate the formation of compact aggregates [4].

Up to now, few researches had investigated the effect on the granulation with different VFA as carbon sources in denitrification.

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Table 1
Experimental settings.

Number	R1	R2	R3	R4	R5	R6
Nitrate	+	+	+	−	−	−
Substrate	Acetate	Propionate	Butyrate	Acetate	Propionate	Butyrate

+ means the reactor with addition of nitrate.
− means the reactor without addition of nitrate.

It was assumed that types of VFA had a significant effect on sludge granulation due to the fact that different VFA had different capacities to produce electron donor for denitrification. This study aimed to remit the accumulation of organic acids and enhance the sludge granulation by adding nitrate in anaerobic process. The relationship between anaerobic granulation and denitrification with different VFA as substrates was investigated. We hope to provide an efficient method to enhance the anaerobic treatment of the high-strength organic wastewater.

2. Materials and methods

2.1. Sludge and wastewater

Sludge was acquired from a secondary sedimentation tank of a local municipal wastewater treatment plant based on the activated sludge process in Dalian, China. The sludge was cultured in a batch anaerobic reactor with a glucose solution (chemical oxygen demand [COD]: 1000 mg/L) as substrate. The sludge was not exposed to nitrate prior to adding into the digesters. The ratio of volatile suspended solids to total suspended solids (VSS/TSS) of the sludge was 0.65.

An artificial wastewater consisted of a single VFA like acetate, propionate and butyrate in turn was used as the influent. NH_4Cl and KH_2PO_4 were used as nitrogen and phosphorus sources, respectively (at mass ratio of COD:N:P 200:5:1). The trace elements including Zn, Mn, Cu, Co, Ni etc., were added according to reference [20].

2.2. Batch experiments

Several same conical flasks with a volume of 250 mL were used as anaerobic digesters. 100 mL sludge with 25 g/L of TSS was added into each digester. 150 mL artificial wastewater with nitrate and a type of VFA (sodium salt of acetate, propionate or butyrate, respectively) was added into each digester as shown in Table 1. The experiment was divided into three stages according to the different composition in the feeding liquid: (1) NO_3^--N of 130 mg/L and VFA of 1000 mg/L (1–30 cycles); (2) NO_3^--N of 260 mg/L and VFA of 1000 mg/L (31–65 cycles); (3) NO_3^--N of 260 mg/L and VFA of 3000 mg/L (66–95 cycles).

The digesters were operated in a semi-continuous (i.e., sequencing batch) mode with a cycling time of 24 h. They were placed on a constant temperature breeding shaker (ZHWY-2102C, China) at 35 ± 1 °C and 120 rpm. After each cycle, the treated liquid was completely poured out and replaced using fresh artificial wastewater. The batch experiments were continuously conducted for 95 cycles. The concentrations of nitrate and VFA were analyzed 3 h after the beginning of each cycle, and the sludge size and biogas content were measured after each cycle. The digesters were flushed with N_2 for 20 min to remove dissolved oxygen, and then sealed with plugs before each cycle.

2.3. Analytical methods

NO_3^--N, pH, TSS and VSS were measured according to the standard methods [27]. The concentrations of VFA, including acetate, propionate, butyrate, were determined using a gas chromatograph (Shimadzu, GC-2010/FID, Japan). The composition of biogas was analyzed by a gas chromatograph (Shimadzu, GC-14C/TCD), Japan). The size distribution of sludge was measured and calculated by Malvern Mastersizer 2000 laser particle size analyzer (Worcestershire, UK) according to the method by Liu et al. [16]. Morphology of granules was examined by a scanning electron microscopy (SEM, Quanta 200 FEG). The granules samples were firstly immobilized using 205% (w/v) glutaraldehyde in Sorenson's phosphate buffer, and dehydrated using ethanol with gradient concentrations (10%, 25%, 50%, 75%, 90% and 100%, 15 min per step). And then the samples were dried using carbon dioxide at their critical point. At last, the samples were sputter coated with gold and observed using the SEM.

2.4. DNA extraction, PCR amplification and denaturing gel gradient electrophoresis

After the experiment (for 95 cycles), approximately 1 mL the sludge samples were collected from the homogenized sludge in each digester. The genomic DNA of the sample was extracted using an extraction kit (Bioteke Corporation, Beijing, China) according to the manufacturer's instructions, before which the sludge samples were firstly washed with phosphate-buffered saline (pH 7.4). The general primers of 341f (5′-CCT ACG GGA GGC AGC AG-3′) which contained a 40 bp GC clamp at the 5′-end (forward primer) and 907r (5′-CCG TCA ATT CMT TTR AGT TT-3′, reverse primer) were used to selectively amplify the 16 S ribosomal RNA sequences of Eubacteria [17]. Polymerase chain reaction (PCR) amplification was conducted on a thermal cycler (Thermal Cycler DiceTM; BioRad Co., Ltd., Hercules, CA, USA) with a touchdown PCR method as following program (initial denaturation at 95 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 30 s, primer annealing at 50 °C for 1 min and primer extension at 72 °C for 2 min, and final extension at 72 °C for 10 min). The PCR products obtained were applied to (denaturing gradient gel electrophoresis) DGGE analysis using the Dcode system (BioRad Co., Ltd., USA). A denaturing gradient of 30–60% denaturant (100% denaturant was a mixture of 7 mM urea and 40% [v/v] formamide) acrylamide gel (6%, w/v) was applied. Electrophoresis was conducted at 60 °C and at a constant voltage of 200 V for 5 h in 1 × TAE buffer. The gels were then stained with SYBR Gold (Dalian TaKaRa, China) in 1 × TAE buffer for 40 min, after which the UV thansillumination image of the gel was then photographed using the Gel Doc 2000 System (BioRad Laboratories, USA). Selected DGGE bands were excised and re-amplified by PCR using the primers described above without the GC clamp. The obtained gene sequences were screened against the GenBank database using the BLAST program to identify the most similar sequences. The DGGE analysis above was conducted according to the reference of Zhang et al. [17].

2.5. Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) was used to determine the abundance of VFA-oxidizing bacteria. The homogenized sludge samples collected from different digesters were harvested by centrifugation (110 × 100 g for 15 min at 4 °C) and fixed in 4% freshly prepared paraformaldehyde solution for 2 h at 4 °C. Then the samples were washed twice with phosphate-buffered saline (PBS, 130 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2) and air dried at room temperature. After that the samples were dehydrated by successive passages through 50%, 80%, and 100% ethanol (three times). Hybridization steps were performed at 46 °C for 1.5 h with buffer (0.9 M NaCl, 20 mM Tris-HCl [pH 7.2], 0.01 sodium dodecyl sulfate and 35% formamide) containing 50 ng probe per microliter and then washed with buffer (15 min at 48 °C).

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