Effects of Calcium Concentration on Up-flow Multistage Anaerobic Reactor Performance in Treating Bagasse Spraying Wastewater

Jinghong Zhou,[a,*] Xiaona Shang,[a] Zhiwei Wang,[a] Cancan Zhu,[a] and Shuangfei Wang [a,b]

Bagasse spraying wastewater (BSW) is a source of organic pollutants during bagasse processing. In this study, the feasibility of anaerobic treatment of BSW under different calcium concentrations (60 to 2400 mg/L) was studied. The experiment was performed in a lab-scale up-flow multistage anaerobic reactor (UMAR) inoculated with granular sludge, and operated for 160 days at a constant organic loading rate of 6 kg COD/(m^3·d). Treatment of BSW with 60 to 800 mg Ca^{2+}/L resulted in 80.7 to 82.7% of COD removal, 161 to 232.7 mg COD/L of volatile fatty acid (VFA) yield, 0.56 to 0.79 m^3/(kgCOD·d) of biogas production rate, and 2.4 to 2.66 m^3/(m^3·d) of volume loading rate (VLR). The pH remained within the optimal range for anaerobic digestion (adjust to pH = 6.8 to 7.0). The VFAs were composed of 77 to 85% acetic acid, 8.4 to 13.2% butyric acid, and 6.6 to 9.6% propionic acid. At higher influent calcium concentrations (> 800 mg/L), the hydrolysis process appeared to be inhibited, affecting the anaerobic digestion performance of the reactor. In particular, the COD removal efficiency decreased to 55.5%, and the VFA content in the effluent significantly increased due to the lower pH. Microbial community analysis showed that at the end of anaerobic digestion, the *Syntrophobacter* disappeared, and *Clostridium* and Anerolineaceae were the main genus and family, respectively. Overall, the results indicated that low calcium (< 300 mg/L) had a positive effect on the UMAR performance.

Keywords: Anaerobic granular sludge; Bagasse spraying wastewater; Calcium; UMAR; Up-flow multistage anaerobic reactor

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INTRODUCTION

China is the world's third largest producer of sugar after Brazil and India. According to a report from the National Bureau of Statistics of China (NBSC 2014), there are approximately 1.7 million ha of sugarcane plantations in southern China, over 60% of which are located in Guangxi Province. Bagasse is an industrial byproduct generated by sugar mills. Every year, more than 20 million tons of bagasse is produced in China, and it is used as one of the main raw materials in paper production. Bagasse is deposited in the open air. In order to protect fibers preferably, improve the quality of bagasse pulp, and reduce alkali charge in cooking process, bagasse is kept by the wet stacking process. This means spraying water regularly in the process of stacking, and conducting a pressing operation to exclude internal gas. The resulting wastewater is referred to as bagasse spraying wastewater (BSW) (Shen *et al.* 2013). BSW contain large amounts of high concentration organic pollutants because of the degradation of the residual sugar. There are three main factors affecting the COD content of BSW: residual sugar in bagasse, stacking time of bagasse, and seasonal weather variations. The concentration of residual sugar changes during biological oxidation, and thus results in a high COD between 6000 and 12,000 mg/L and low pH (2 to 3) (Nie *et al*. 2005; Yang 2013).

Anaerobic treatment is the most promising and useful technology to treat high strength organic wastewaters (Frankin 2001; Pol *et al*. 2004; Zhang e*t al*. 2011). In anaerobic processes, the methane organisms function over a pH range of 6.6 to 7.6 with an optimum near pH 7.0. When the rate of acid formation exceeds the rate of breakdown to methane, a process unbalance results in which the pH decreases, gas production falls off, and the CO_2 content of the gas increases (Dolfing 1986; Gerardi 2006). Using pH control is therefore essential to ensure a high rate of methane production. In China, in the process of anaerobic treating BSW, lime is commonly used to raise the pH of the anaerobic system, resulting in high calcium concentrations in BSW. The effect of calcium addition on anaerobic digestion showed that calcium has been found to be moderately inhibitory at concentrations of 2.5 to 4 g/L and strongly inhibitory at 8 g/L (Parkin and Owen 1986; Langerak 1998). However, another study on the treatment of synthetic wastewater showed that the addition of calcium concentrations up to 7 g/L had no inhibitory effect on the methanogens (Jackson-Moss *et al.* 1989). Thus, in the industrial wastewater treatment, the effect of calcium addition on anaerobic wastewater treatment is not clearly understood.

The up-flow multistage anaerobic reactor (UMAR) has been used to treat cassava starch wastewater (Sun *et al.* 2012). Zhou *et al.* (2009) employed UMAR treating molasses alcohol wastewater and found the maximum 81% COD removal at hydraulic retention time (HRT) of 7.1 h and the organic load (OLR) of 17.6 to 33.8 kg/(m^3·d) at a fixed COD of 10000 mg/L. However, no studies have considered the effect of calcium on the efficiency of UMARs in treating BSW. Therefore, the objective of this research was to evaluate the operational characteristics of UMAR during treatment of BSW with high calcium concentration. A laboratory-scale UMAR was operated for 160 days to evaluate the effects of calcium on the long-term performance of the UMAR in treating BSW. The effects of a constant organic loading rate (OLR) under various influent calcium concentrations (60 to 2400 mg/L) were examined to determine the influence of calcium concentration on the COD removal efficiency, volatile fatty acid (VFA) yield and composition, biogas production and composition, and sludge characteristics.

EXPERIMENTAL

Experimental Setup and Operation

Figure 1 presents a schematic diagram of the laboratory-scale UMAR used in this study. The setup included a BSW feed tank, pH control unit, wet gas flow meter, and the main UMAR vessel. The effective volume of the UMAR was 12.6 L with an internal diameter of 12 cm and a reaction zone height of 112 cm.

Gas–liquid–solid separators avoid anaerobic sludge washout and prevent loss from the reactor. The first gas–liquid–solid separator was installed at the top and second gas–liquid–solid separator was located in the upper middle of the UMAR. The gas–liquid separator and the first and second gas–liquid–solid separators were connected to the sludge expanded bed with a gas riser pipe and a liquid down-flow pipe. Biogas generated during the anaerobic process was collected by the gas–liquid–solid separators and stored in the biogas tank. Liquid was refluxed to the bottom of the reactor through the liquid down-flow pipes as a result of internal circulation. After primary treatment of wastewater through the first gas–liquid–solid separator, the liquid entered the secondary treatment area for further processing. Sampling ports were installed every 10 cm along the reactor height. The reactor was inoculated with 5 L of granular sludge. The OLR was set at 6 kg COD/(m^3·d) with an HRT of 24 h as the start-up conditions. The biogas produced in the UMAR was first passed through a bottle filled with a 3% NaOH solution to adsorb carbon dioxide, hydrogen sulfide, and other trace gases, and methane production was volumetrically measured using a gas meter (TC-LMF-1; Beijing; China).

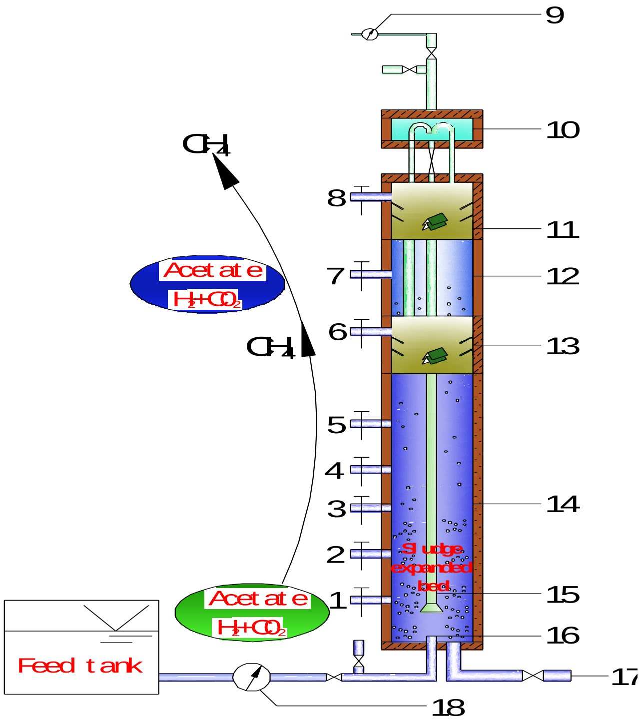

Fig. 1. Schematic diagram of the up-flow multistage anaerobic reactor: (1–8) sampling and effluent ports; (9) gas-flow meter; (10) gas–liquid separator; (11) second gas–liquid–solid separator; (12) primary treatment; (13) gas riser pipe; (14) first gas–liquid–solid separator; (15) sludge expanded bed; (16) down-flow pipe; (17) distributor; (18) effluent port; (19) pump

Wastewater Composition

The BSW was provided by Nanning Paper Co., Ltd. (Guangxi, China). The characteristics of the wastewater used in this study are given in Table 1. The BSW was acidic (pH 2.0 to 3.0), and the biological oxygen demand (BOD)/COD ratio of 0.47 supported the suitability of biological treatment for the wastewater.

Table 1. Characteristics of the Bagasse Spray Wastewater

Parameter	pH	Chemical Oxygen Demand (mg/L)	Biological Oxygen Demand, 5-day (mg/L)	Suspended Sediment (mg/L)	Total Nitrogen (mg/L)	Total Phosphorus (mg/L)	Ca^{2+} (mg/L)
Value	2–3	6000–12000	4000–6000	50–70	90	150	60–80

Seed Sludge Source

The anaerobic granular sludge used in the experiment was obtained from the wastewater treatment plant of the Nanning Paper Mill (Guangxi, China). The morphology of the granular sludge was flat and spherical or oval with a particle size of 2 to 4 mm, wet density of 1.05 kg/m^3, and sedimentation rate of 50 to 70 m/h. The pH of the sludge was 7.1 to 7.3, and the ratio of volatile suspended solids to total suspended solids (VSS/TSS) was 67.12%.

Analytical Method

COD, BOD, suspended solids (SS), total nitrogen, and total phosphorus, TSS, and VSS were measured in triplicate according to standard methods (Walter 1998). The concentrations of VFAs, including acetic, propionic, and butyric acids, were determined using gas chromatography (GC; 6890N, Agilent, Palo Alto, CA, USA). The GC conditions were as follows: DB-FFAP chromatography column (30 m × 0.25 mm × 0.25 µm), initial temperature of 80 °C, increased to 100 °C at 5 °C/min, to 136 °C at 6 °C/min and, finally, to 800 °C at 8 °C/min; gasification chamber temperature of 100 °C; hydrogen flame detector (FID) temperature of 250 °C. HS conditions were as follows: constant temperature box temperature of 50 °C and sample equilibration time 5 min (Chai *et al.* 2006; Sun *et al.* 2007). Similarly, methane and carbon dioxide concentrations were measured using a GC system equipped with a fully automatic top space sampler (G1888A; Agilent). The test sample was injected into the GC with an air-tight syringe, and GC conditions: GS-Q chromatographic column (Agilent, USA), temperature 60 °C, carrier gas is nitrogen, flow rate of 104 mL/min, injection port temperature 200 °C, FID hydrogen flow rate of 40 mL/min, air flow rate of 400 mL/min, and detector temperature 250. The HS conditions were maintained with a constant temperature box at a temperature of 37 °C. The sample bottle was in a low-speed shaking state, and samples were taken at an interval of 20 min. During sampling the pressure time kept was 0.10 min, the sample injection time was 1 min, the sample filling time was 0.10 min, and the sample ring equilibrium time was 0.05 min (Chai *et al.* 2006; Sun *et al.* 2007).

The specific methanogenic activity (SMA) assay is a direct method used to test the methanogenic activity of granular sludge, and it is determined by measuring the methane production rate of a sludge sample. The biogas volumes were measured at 25 °C, and the dissolved gas compositions were determined using the headspace method (Bandara *et al.* 2011). The pH was determined using a pH electrode.

The calcium concentration (in both particulate and soluble forms) was measured with inductively coupled plasma atomic emission spectroscopy (ICP-AES; 5300DV, Agilent). Effluent samples were filtered through a 0.45-µm PES membrane filter. To analyze the particulate calcium, sludge samples were dried (105 °C), solubilized by adding 6 mL nitric acid, 2 mL hydrochloric acid, 3 mL hydrofluoric acid, and 1 mL hydrogen peroxide, and then heated by microwave digestion. After cooling, 3 mL of perchloric acid was added and the samples were diluted with demineralized water to a volume of 100 mL. Before analysis, all samples were diluted with 5% nitrate solution (Liu *et al.* 2011).

The anaerobic granular sludge apparent structure and microbial was observed by scanning electron microscopy (SEM; Hitachi S-3400N, Hitachi, Japan). The observations were carried out as described previously (Fukuzaki *et al.* 1991).

Microbial Community Structure Analysis

Sample A is control sludge without added calcium ions. Sample A was taken from the inoculated sludge blank, and deionized water replaced BSW in the domestication process, while B, C, D, E, F, G, and H were collected from the reactor on the fifth day and the end of the experiment of each phase (with 60, 300, 800, 1200, 1600, 2000, 2400 mg/L of added $CaCl_2$, respectively). The DNA extraction was performed according to the E.Z.N.A. soil DNA kit method (Omega Biotek, Norcross, GA, USA). A 1% agarose gel electrophoresis was used for the detection of extracted DNA quality, while the concentration and purity were detected with NanoDrop2000 (USA). PCR amplification using 338F (5'-GGACTACHVGGGTWTCTAAT-3') and 806R (5'-ACTCCTACGGGA-GGCAGCAG-3') was carried out on the V3-V4 variable area. PCR products were recovered using an AxyPrepDNA Gel Extraction Kit purification (Axygen Biosciences, Union City, CA, USA), eluted with Tris-HCl buffer, and detected by 2% agarose gel electrophoresis. Using QuantiFluorTM-ST (Promega, Madison, WI, USA) to quantitative detection, and according to the standard operating procedures of the Illumina MiSeq platform (Illumina, San Diego, CA, USA), purified fragments were amplified and built into the PE2 * 300 library (Wang *et al.* 2018).

Experimental Method

The influent calcium concentration was increased in steps, from 60 to 300 mg/L (day 23), 300 to 800 mg/L (day 46), 800 to 1200 mg/L (day 80), 1200 to 1600 mg/L (day 100), 1600 to 2000 mg/L (day 118), and 1600 to 2400 mg/L (day 138), at a fixed OLR of 6 kg COD/(m^3·d) over the 160-day operation. Before adding different calcium concentrations, the UMAR was allowed to achieve stable operation, during which period the average COD removal efficiency exceeded 80%. During the acclimation process, the influent pH was adjusted to the range 6.8 to 7 with sodium hydroxide, and the COD concentration was held constant at approximately 6000 mg/L throughout the acclimation period at a fixed HRT of 24 h. To examine the effects of calcium on COD removal, gas production, and sludge granulation, an initial calcium concentration of 60 mg/L was added to the BSW. After increasing the calcium concentration, the effluent COD removal efficiency stabilized after approximately 15 days, after which the influent calcium concentration was further increased.

RESULTS AND DISCUSSION

Effects of Calcium Loading on COD Removal Efficiency

Granular sludge was incubated in the UMAR for 160 days. The effect of the influent calcium concentration on COD removal was investigated (Fig. 2). The average

COD removal efficiency was 80.7% on day 22 of operation. Calcium showed a positive effect on the COD removal efficiency during the initial increase. For instance, after the influent calcium concentration was increased from 60 to 300 mg/L on day 23, the COD removal efficiency greatly increased to 94.4% on day 44. However, after all subsequent increments in calcium concentration, the COD removal efficiency decreased and high calcium concentrations had an adverse effect on COD removal. For example, at 800 mg Ca^{2+}/L, the average COD removal efficiency significantly decreased to 71.8%. Finally, at 2400 mg Ca^{2+}/L, the average COD removal efficiency decreased to 55.5%. Therefore, calcium concentrations up to 300 mg/L had a positive effect on the treatment of high-COD BSW.

The results in the present study appeared to have been caused by the calcium concentration in the BSW, as increasing the influent calcium concentration resulted in an increase in the concentration of calcium in the granular sludge. This could have resulted in calcium toxicity causing inhibition of anaerobic granular sludge activity and, subsequently, anaerobic digestion. In addition, the introduction of calcium increased the hardness of the entire anaerobic system, thereby enhancing autolysis of bacterial cells and causing the release of large quantities of cellular components (Sivaprakasam *et al.* 2008). Because the release of cellular components is a continuous process, it would result in a low degradation rate.

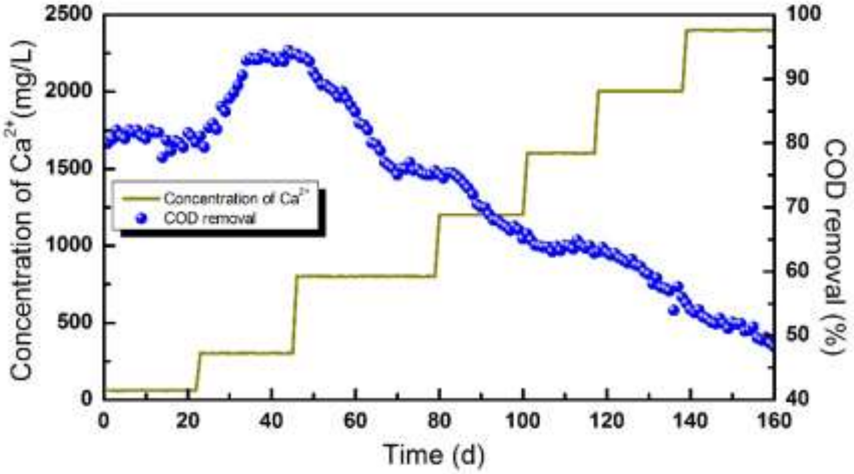

Fig. 2. Effect of calcium ion concentration on the chemical oxygen demand (COD) removal efficiency of the up-flow multistage anaerobic reactor

Effect of Calcium Loading on VFAs and pH

The pH has a significant role in the anaerobic digestion process. Acidogenic populations are significantly less sensitive to pH fluctuations than methanogenic populations, which require the pH of 6.5 to 7.5. Acid formation prevails over methanogenesis when the pH is low, resulting in an accumulation of VFAs in the reactor (Kuruti *et al.* 2017). The VFAs, such as formic, hexanoic, acetic, propionic, and butyric acids, are important intermediates of methane production during anaerobic degradation. The VFA composition can provide useful information regarding the degree of hydrolysis and fermentation.

At low calcium concentrations (60 to 800 mg/L), the pH remained approximately constant at a mean value of 7.0, within the optimal range for anaerobic digestion (Fig. 3). The VFA yield was in the range of 161.2 to 232.7 mg COD/L, and the main VFA components were acetic acid (85.0 to 77.2%), butyric acid (6.6 to 9.6%), and propionic acid (8.4 to 13.2%). However, at higher calcium concentrations, the VFA yield increased. When the calcium concentration increased from 800 to 1600 mg/L, the VFA content in the effluent dramatically increased from 232.7 to 332.5 mg COD/L. As the calcium concentration increased to 2400 mg/L, the VFA content further increased to 514 mg COD/L. Thus, the VFA content in the effluent greatly increased. At the same time, the VFA composition also changed. When the calcium concentration increased from 800 to 2400 mg/L, the proportion of acetic acid decreased from 77.2 to 52%, while the propionic acid and butyric acid proportions increased from 9.6 to 21% and 13.2 to 27%, respectively. At calcium concentrations exceeding 800 mg/L, the pH gradually declined from 7.2 to 7.4 to 6.7 to 7.1 due to acid accumulation in the reactor. VFA levels in UMARs can affect the pH, which can, in turn, affect the types of VFAs (*e.g.*, acetic, propionic, and butyric acids) produced via acidogenic fermentation (Bengtsson *et al.* 2008). Under high VFA production, the pH of the effluent significantly decreased, similar to the trend in VFA composition and pH observed by Ren *et al.* (2007). In the present study, when calcium concentrations varied within an appropriate range, the VFAs could rapidly be digested by methanogenesis. However, under excess calcium concentrations, the VFAs failed to be consumed by methanogenes in a timely manner, leading to excessive VFA accumulation and a severe decline in pH, which could inhibit methane production.

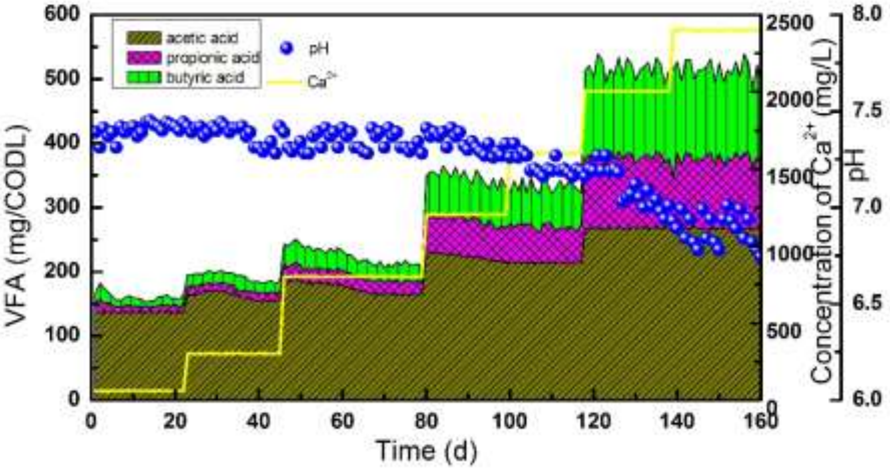

Fig. 3. Effect of the calcium ion concentration on the volatile fatty acid (VFA) composition and pH of the up-flow multistage anaerobic reactor

Biogas Production Rate and VLR in the UMAR

The VLR and biogas production rate are two important physical parameters for characterizing the operation state of anaerobic reactors. The volume loading rate (VLR) was determined to examine the methane produced per unit volume of the anaerobic reactor per unit time, while the biogas production rate was determined to examine the methane produced per unit of degraded organic load in the anaerobic reactor. Higher VLRs are indicative of better anaerobic methane production performance, and higher methane production is indicative of a more suitable environment for anaerobic methane production.

The biogas production rate and VLR of acclimated anaerobic granular sludge samples were tested at different calcium concentrations over 160 days (Fig. 4). In the initial stages, the biogas production rate and VLR increased as the calcium concentration increased. At low calcium concentrations (< 300 mg/L), the biogas production rate and VLR reached maximum values of 0.59 m^3/kg COD·d and 3.20 $m^3/(m^3 \cdot d)$, respectively. With the calcium concentration further rising to 800 mg/L, the VLR fell to 2.66 $m^3/(m^3 \cdot d)$ and the biogas production rate was 0.79 m^3/kg COD·d. As the calcium concentration increased to 1600 mg/L and 2400 mg/L, the biogas production rate gradually decreased to 0.33 m^3/kg COD·d and 0.40 m^3/kg COD·d, respectively. Liu *et al.* (2011) found that the calcium accumulation onto the granules was monotonically related to the calcium concentration. High calcium concentrations in granules could damage the environment required to maintain the granular structure or bacterial activity and possibly inhibit the VLR (Yu *et al.* 2001, Liu *et al.* 2011, Dang *et al.* 2014). From the experimental results (Fig. 4), it was apparent that when calcium concentration was 800 mg/L, the methane production was much lower than that of 300 mg/L, and the SMA was reduced to 2.79 $gCOD_{CH4}/(g\ VSS \cdot d)$ (Fig. 5). This indicated that under these calcium concentrations, the SMA of granular sludge was slightly inhibited. High calcium concentrations significantly inhibited methanogenic activity, while the carbon dioxide production rate was promoted. The production of methane decreases while that of carbon dioxide increases (Fig. 5).

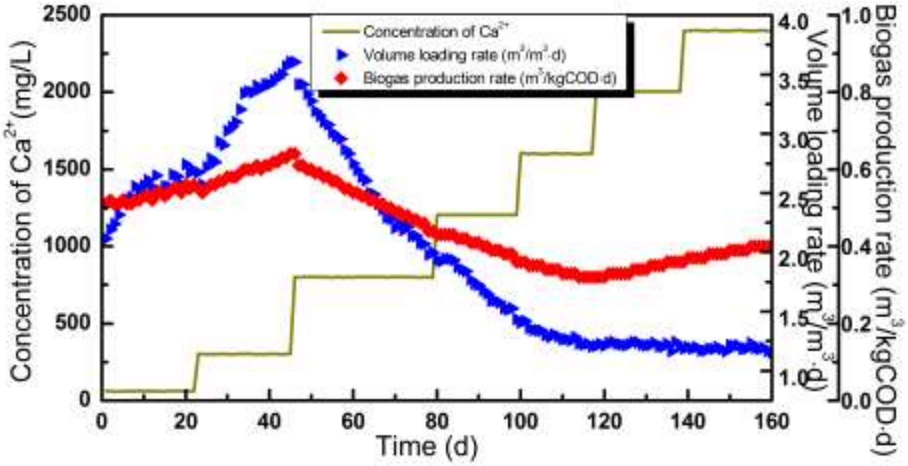

Fig. 4. Biogas production rate and volume loading rate of the up-flow multistage anaerobic reactor

Effect of Calcium Concentration on SMA and Biogas Composition

The SMA was used to characterize the methanogenic activity of the anaerobic sludge. Figure 5 presents the SMA and biogas composition of granular sludge under different calcium concentrations. As shown in Fig. 5, when the influent calcium concentration was gradually increased from 60 to 300 mg/L, the CH_4 percentage in the produced biogas was quite constant, mainly in the range of 65 to 70%, while the CO_2 percentage was 30 to 35%. This showed that the methanogenic activity of granular sludge was very stable at low calcium concentration (< 300 mg/L). When the calcium concentration was 300 mg/L, the SMA of the anaerobic sludge reached the maximum of 5.96 $gCOD_{CH4}/(g\ VSS \cdot d)$. However, when the calcium concentration increased from 300 to 800 mg/L, the SMA decreased by 2.79 $gCOD_{CH4}/(g\ VSS \cdot d)$, the methane proportion decreased from 67.1 to 60.0%, and the carbon dioxide proportion increased from 32.9 to 40%. Therefore, 800 mg/L of calcium slightly inhibited methanogenic activity. Moreover, when the calcium concentration increased from 800 to 2400 mg/L, the methane gas proportion decreased from 47.6 to 6.9%, the carbon dioxide proportion significantly increased from 52.4 to 93.1%, and the SMA decreased to 0.34 $gCOD_{CH4}$/(g VSS·d).

The above results showed that in the initial granulation period (calcium concentration < 300 mg/L), calcium had a positive effect in enhancing the SMA of the granular sludge, similar to a report by Ahn *et al.* (2006). However, as the calcium concentration increased, especially to 1200 to 2400 mg/L, it exerted a negative effect, reducing the sludge SMA and methane production rate. Therefore, low calcium concentrations can promote the methanogenic activity of granular sludge, whereas high calcium concentrations can inhibit methanogenic activity (Dang *et al.* 2014). Moreover, previous reports have revealed other problems, including blockage of the inlet flow by sludge settling and scale formation on the outlet pipes due to floating organic calcium species, which are problematic for the practical operation of up-flow anaerobic sludge bioreactors (Langerak *et al.* 1998; Yong *et al.* 2004; Pol 1989).

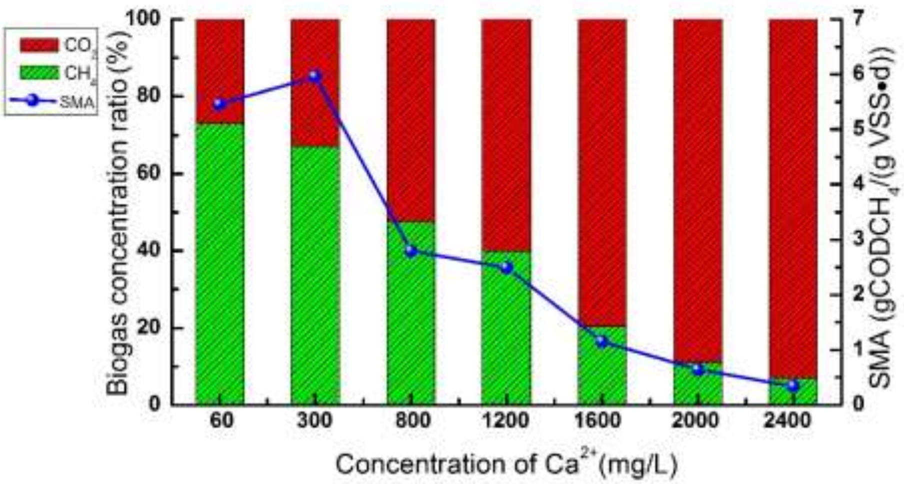

Fig. 5. Effect of calcium ion concentration on the sludge methanogenic activity (SMA) and biogas composition.

Calcium Concentration in Anaerobic Granular Sludge

Calcium has an important role in anaerobic granular sludge formation, as it affects the sludge concentration, sludge settling velocity, granular strength, and treatment efficiency of the anaerobic reactor (Abbasi and Abbasi 2012; Wang *et al.* 2014). The calcium concentrations in the granules taken from sampling ports 1, 3, 5, and 7 in the UMAR were nearly proportional to the calcium concentration in the substrate (Table 2). At relatively low calcium concentrations (60 to 300 mg/L) in the influent, there was no obvious calcium precipitation on the sludge granules, and the calcium weight percentage remained relatively constant. However, high calcium concentrations (800 to 2400 mg/L) caused faster accumulation on sludge granules. At such concentrations, calcium accumulation within the granules was mostly driven by the interactions of calcium ions with carbonate and exopolysaccharide polymers, because the calcium concentration within bacterial cells is very low (Langerak *et al.* 2000; Alazmi *et al.* 2010). Moreover, the formation of calcium carbonate and calcium bound to exopolymers and cells in granules are nonspecific processes driven by the calcium ion gradient from the bulk liquid phase into the granules (Yu *et al.* 2001). Yu *et al.* (2001) reported the concentration of 150 to 300 mg/L calcium in synthetic wastewater enhanced the biomass accumulation and granulation process. The specific activity of granules decreased with increasing influent calcium concentration. Meanwhile, Langerak *et al.* (1998) reported the lower calcium concentration of 600 mg/L in acidified wastewater with a high COD removal efficiency (98%) and less calcium precipitated.

The reactor-fed BSW with a higher calcium content showed faster calcium accumulation on sludge granules. The factor determining the amount of precipitated calcium was the influent calcium concentration, where higher influent calcium concentrations resulted in higher calcium precipitation. At higher calcium concentrations, mineral precipitates such as calcium carbonate form, running the risk of reactor cementation. Moreover, calcium carbonate precipitates on sludge granule surfaces prevent bacteria from contacting organic matter, and increase the settleability of some large granules to the reactor bottom, making them unavailable for fermentation, and ultimately reducing the biological activity of the reactor (Abbasi and Abbasi 2012).

Table 2. Calcium Concentrations in Sludge Granules (as total suspended solids (TSS)) from Four Sampling Ports (#) in the up-flow Multistage Anaerobic Reactor

Influent Ca^{2+} (mg/L)	Ca^{2+} Concentration in Sludge (g/kg TSS)			
	#1	#3	#5	#7
60	38.47	34.63	30.64	23.84
300	40.87	36.82	33.59	26.05
800	50.74	43.95	39.85	30.83
1600	64.84	59.72	56.82	47.73
2400	79.64	74.29	70.85	62.93

Anaerobic Characterization

The appearance and microbiofacies of the anaerobic granular sludge were observed and analyzed using SEM imaging. Figures 6 and 7 show the granular structure and microbiofacies during the initial stage (calcium ion concentration = 60 mg/L) and at the end of the experiment (calcium ion concentration = 2400 mg/L), respectively. At the beginning of the experiment, the surface of spherical granules was rough with many cavities and cracks (Fig. 6a), which were anticipated to provide substrates and intermediate products access to the inside of the granule and provide an escape route for biogas produced during methanogenesis. The SEM images showed that different types of microorganisms were randomly interwoven throughout the cross-section of granules. A large population of filamentous microorganisms was observed to have formed a network on the surface of the granules (Figs. 6b and c), which appeared to be in a loose formation in the interior of the sludge granules. In addition, many bacilliform bacteria colonies were observed inside the granules, linked together by filamentous microorganisms (Fig. 6d). Overall, the filamentous microorganisms and bacilliform bacteria were present on the surface, inner layer, and core of the granules. Based on a report by Zinder *et al.* (1984), such filamentous microorganisms had morphologies typical of *Methanothrix* spp.

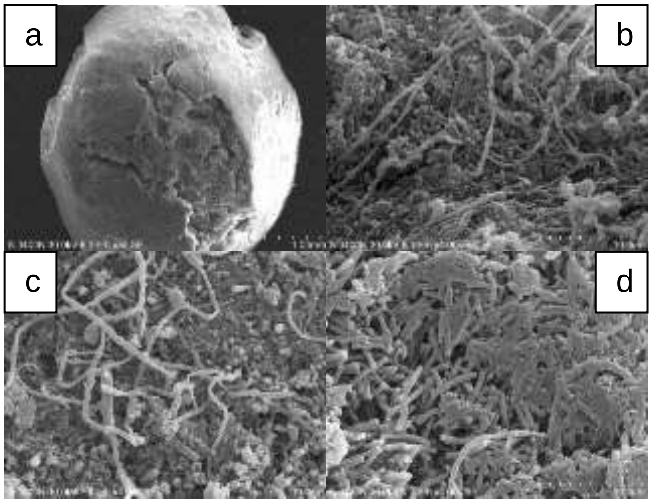

Fig. 6. Microorganisms on the granular sludge surface (calcium ion concentration = 60 mg/L): (a). cemented sludge from the bottom part of up-flow multistage anaerobic reactor at the start of the experiment, which consisted of spherical particles; (b, c) filamentous microorganisms on the surface of the granules; (d) *Bacillus* colonies observed inside the granules

At the end of the experiment (calcium ion concentration = 2400 mg/L), the granule samples presented different morphological characteristics. For instance, the surface of the particles had a dense crystal structure (Fig. 7a). Moreover, agglomeration of surface crystals due to accumulated calcium carbonate was observed. Fewer bacteria were present in the inner granules or it was due to the accumulation of calcium. Hence it was difficult to observe the bacteria present. There were a large number of random solids (Figs. 7b, c, and d), which may have affected the mass transfer of anaerobic bacteria, thus affecting the biological phase of anaerobic sludge. In addition, Langerak *et al.* (1998) reported the calcium effect on the development of methanogenic sludge in UASB, these microorganisms had similar morphological characteristics.

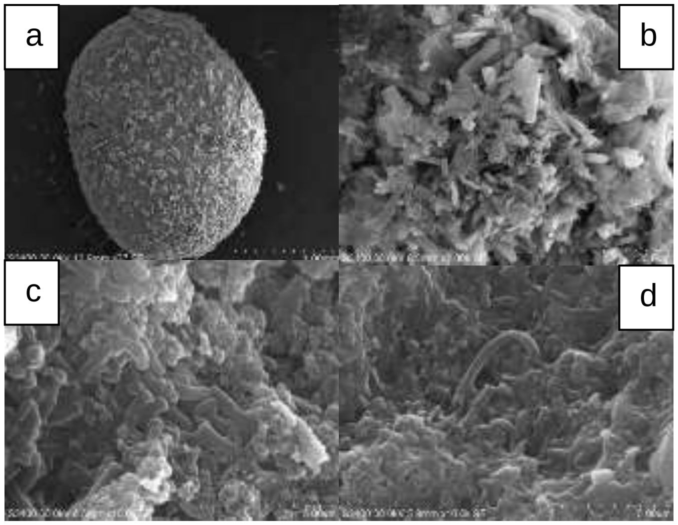

Fig. 7. Microorganisms on the granular sludge surface (calcium ion concentration = 2400 mg/L): (a) granule from the bottom of up-flow multistage anaerobic reactor at the end of the experiment, showing the calcium-dense crystal surface of particles; (b) a large amount of random solids on the surface; (c, d) relatively few bacteria within the granules.

Microbial Community Analysis

To identify the microbial community structure, the genus of microbes in anaerobic digestion sludge was analyzed. *Syntrophobacter* can degrade the acetate and proinate to CO_2 and H_2 (Liu *et al.* 2018). Anaerolineae can degrade macromolecules in the methanogenic biological system and is the dominant bacterium in the system (Narihiro *et al*. 2009). Figure 8 shows that Anerolineaceae, *Clostridium sensu stricto_1*, and *Syntrophobacter* were the dominant domain in the sample on the fifth day of each phase, *i.e.*, with A–H representing added calcium of 0, 60, 300, 800, 1200, 1600, 2000, and 2400 mg/L, respectively. As the anaerobic digestion process progressed, the proportion of Anaerolineae in the total sequence gradually decreased. *Clostridium X* is a strict class of anaerobic bacteria that can degrade carbohydrates to produce products such as acetic acid and butyric acid (Narihiro *et al.* 2009; Guo *et al.* 2015). The genus *Clostridium sensu stricto*_1 accounted for 7.24, 6.58, 6.62, 17.49, 10.20, 15.86, 12.76, and 19.50% of each phase on the fifth day, respectively. In terms of performance, *Clostridium sensu stricto*_1 scored higher for each phase, accounting for 26.14, 19.84, 23.85, 22.43, 19.96, 14.87, 11.50, and 9.14%, respectively. In terms of performance at the end of each phase *Clostridium sensu stricto_1*, *Clostridium sensu stricto_3*, and *Anerolineaceae* were the dominant in the sample (Fig. 9). The result shows that, at the genus level, with the anaerobic digestion process progressed, calcium ions can inhibit the activity of *Syntrophobacter* bacteria.

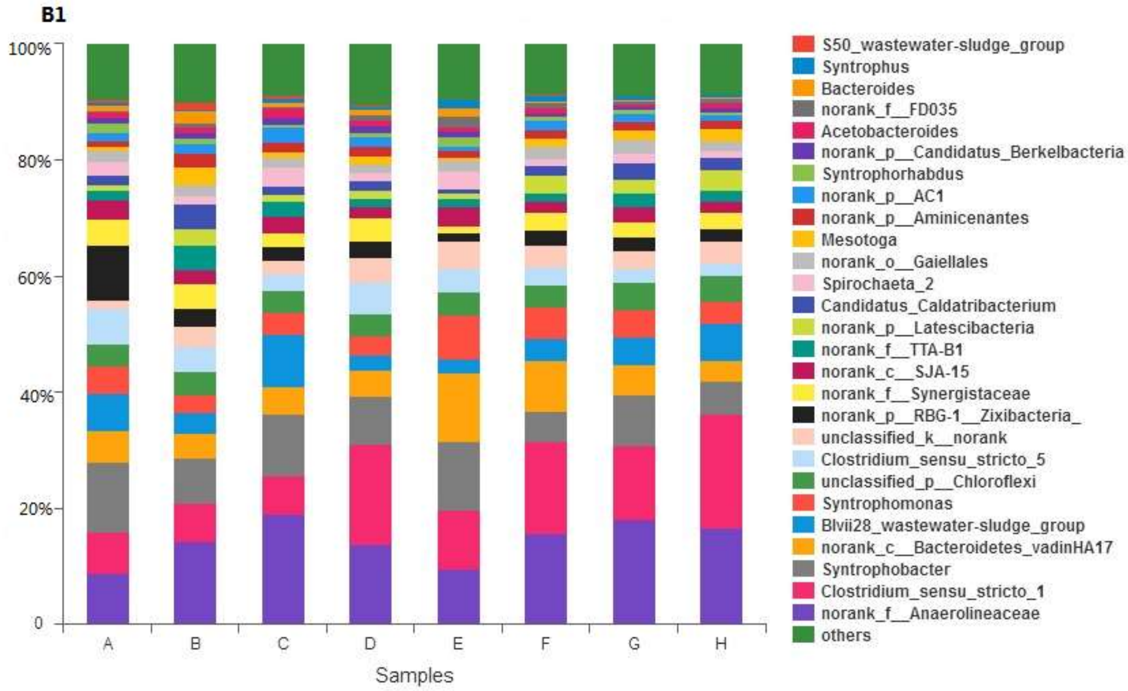

Fig. 8. Genus-level distribution of microbial community on the fifth day of each phase: A, 0 mg/L; B, 60 mg/L; C, 300 mg/L; D, 800 mg/L; E, 1200 mg/L; F, 1600 mg/L; G, 2000 mg/L; H, 2400 mg/L

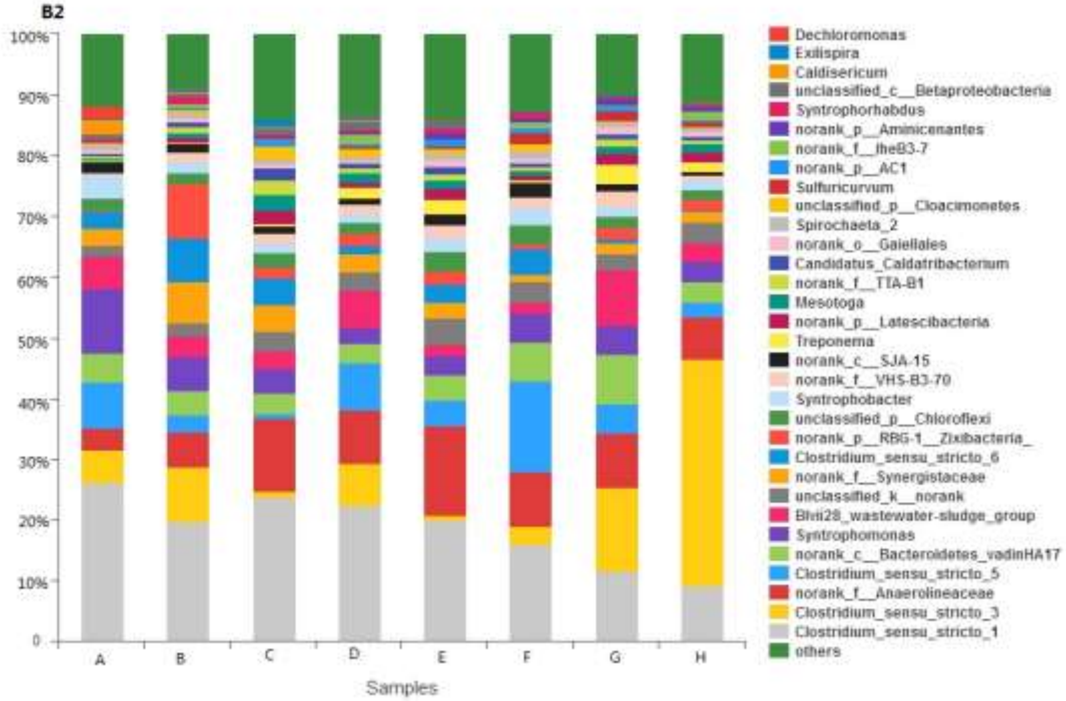

Fig. 9. Genus-level distribution of microbial community at the end of each phase of the experiment: A, 0 mg/L; B, 60 mg/L; C, 300 mg/L; D, 800 mg/L; E, 1200 mg/L; F, 1600 mg/L; G, 2000 mg/L; H, 2400 mg/L

CONCLUSIONS

1. The effects of calcium concentration on reactor performance and granular sludge properties were investigated using an up-flow multistage anaerobic reactor (UMAR) to treat bagasse spraying wastewater (BSW). High calcium concentration leads to the decrease of biological activity and the precipitation of calcium in granular sludge, which leads to the decrease of treatment efficiency.
2. The composition of produced volatile fatty acid (VFAs) was significantly affected by calcium concentration. High calcium concentration (Ca^{2+} >300 mg/L) affected the methane activity of granules and resulted in a decrease in methane production rate.
3. The calcium concentration in the sludge granules was nearly proportional to the influent calcium concentration. The specific activity of granules increased with increasing influent calcium concentration under to 800 mg/L, after which it decreased.
4. The findings of this study clarify the threshold for calcium suppression of reactor operation, and can be applied to ensure that influent calcium concentrations are controlled to ensure maximum BSW treatment efficiency.

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