Effect of the Ca^{2+} Concentration on Anaerobic Digestion and Microbial Communities of Granular Sludge

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The effects of substrate Ca^{2+} concentration were studied relative to the anaerobic digestion and microbial community structure of anaerobic granular sludge. In the treatment of papermaking wastewater by anaerobic granular sludge, the cumulative gas production was maximum at a Ca^{2+} concentration in the substrate of 120 mg/L, whereas it was the lowest at a concentration of Ca^{2+} in the substrate of 4000 mg/L. A high Ca^{2+} concentration in the substrate ($\geqslant$1200 mg/L) will cause the pH of the fermentation broth to decrease during the fermentation process, which is not conducive to the anaerobic fermentation of the methanogenic process, resulting in unsatisfactory anaerobic digestion of the granular sludge. In addition, when the Ca^{2+} concentration was below 200 mg/L, the abundance of the important bacterial family Ruminococcaceae (rumen bacteria) in the anaerobic fermentation hydrolysis stage was drastically reduced and methane gas production increased. When the Ca^{2+} concentration was above 200 mg/L, the abundance of Anaerolineaceae (Anaerobic Streptomyces), which supplies organic acids, was substantially reduced. The methane gas production decreased as the Ca^{2+} concentration increased. Thus, the results showed that when the concentration of Ca^{2+} was above 200 mg/L, the methanogenic activity of granular sludge decreased.

Keywords: Anaerobic Granular Sludge; High throughput sequencing; Microbial community; Ca^{2+} concentration

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INTRODUCTION

Since a large amount of $CaCO_3$ is used in the papermaking process to improve the whiteness and opacity, as well as the softness and smoothness of the paper, a relatively high amount of $CaCO_3$ remains in the paper. As a result, the high-concentration organic wastewater generated during the recycling of waste paper will contain a large amount of Ca^{2+}, which may greatly interfere with the degradation of anaerobic microorganisms. The interference of Ca^{2+} on anaerobic digestion has also beset other wastewaters, such as municipal wastewater, pig manure wastewater, and citric acid production wastewater (Xue *et al.* 2015). Researchers involved in the treatment of different wastewaters using different anaerobic reactors also have tried to elucidate the effect of the Ca^{2+} concentration in the substrate on the formation and digestion of anaerobic granular sludge. For instance, Langerak *et al.* (1998, 2000) used Upflow Anaerobic Sludge Bed (UASB) with fully acidified and partially acidified wastewater. The results show that a high concentration of Ca^{2+} (780 mg/L ± 1560 mg/L) in the substrate can result in the

formation of dense particles in anaerobic sludge, so that the removal rate of chemical oxygen demand (COD) in the wastewater is reduced and cementation of the sludge bed has occurred. Yu *et al*. (2001) considered that during the process of the UASB reactor start-up, Ca^{2+} has a direct relationship with the formation of anaerobic granular sludge, where Ca^{2+} with a low concentration of approximately 150 mg/L to 300 mg/L, can accelerate the granulation process of granular sludge. High concentrations of Ca^{2+} ($\geq$ 450 mg/L) lead to the accumulation of large amounts of minerals into granular sludge and reduce the anaerobic capacity of microorganisms (Yu *et al.* 2001).

Previous studies showed that calcium concentration has a certain influence on anaerobic digestion (Ahn *et al.* 2006). Ca^{2+} showed a moderate inhibitory effect on anaerobic digestion when its concentration was 2.5 to 4 g/L, and 8 g/L Ca^{2+} concentration showed a strong inhibitory effect on anaerobic digestion (Parkin and Owen 1986). In addition, 4.8 g/L Ca^{2+} concentration showed moderate inhibition (Speece 1983). In contrast, another study found that 7 g/L Ca^{2+} concentration exhibited no inhibitory effect on methanogens (Jackson-Moss *et al.* 1989).

Currently, there is no consensus on the specific concentration of Ca^{2+} that affects anaerobic granular sludge, and there is little research on the effects of Ca^{2+} concentration on the microbial community structure. This study will establish a certain gradient of Ca^{2+}concentration and uses a mixture of papermaking fiber materials and distilled water as a substrate. Macroscopically, from aspects, such as cumulative gas production and the substrate consumption rate, the Ca^{2+} concentration in the substrate is analyzed for papermaking fibers. Microscopically, high-throughput sequencing was used to analyze the microbial composition of the granular sludge in an effort to obtain certain data for revealing the effect of the Ca^{2+} concentration in the substrate on the degradation of the granular material by the granular sludge.

EXPERIMENTAL

Materials

Plant fiber material

Papermaking fiber raw materials were obtained from a paper mill (Nine Dragons Paper, Dongguan, China). A coniferous pulpwood board (Nine Dragons Paper, Dongguan, China) was crushed, placed in a sealed bag, stored, and sterilized before use. According to the method of Liu (2004), the cellulose, hemicellulose, and ash contents were 67.4%, 18.5%, and 3.7%, respectively.

Anaerobic granular sludge

Anaerobic sludge was collected from a well-functioning anaerobic internal circulation (IC) reaction tower at a paper mill (Hanyang Paper, Wuhan, China). The Ca^{2+} concentration in waste papermaking wastewater was 232 mg/L, and the Ca^{2+} content on the sludge surface was 2.7%. After the removal of most of the water, it was stored in a refrigerator at 5 °C (Han 2008). When re-activated, the granular sludge was intact. Most of it was black, glossy, and spherical, while a small part of it was khaki-colored and spherical. After the sludge was drained, according to the method of Liu (2004), the total solids (TS) and volatile suspended solids (VSS) were 31.7% and 16.4%, respectively.

Experimental device

Anaerobic digestion experiments were performed in 500-mL saline bottles (actual volume capacity of 610 mL) using a disposable infusion set as the airway to connect an inverted saline bottle containing 500 mL of saturated saline and another disposable infusion set as a drain pipe connected to a water bottle. In this experiment a 300-mL conical flask was used as a water bottle. Then, the vial was placed in a constant temperature water bath at 35 °C ± 1 °C.

Experimental process

For the test batch anaerobic digestion, the volume capacity of the anaerobic reaction device was 610 mL, while the working volume was 300 mL. Various calcium chloride solutions with the Ca^{2+} concentrations of 0, 40, 120, 200, 400, 1200, 2000, and 4000 mg/L were prepared and identified as A, B, C, D, E, F, G, and H, respectively. Then, two parallel tests were prepared for each group, with the organic load factor set to 10 $gVS{\cdot}L^{-1}$, with a reaction temperature of 35 °C ± 1 °C, an inoculum ratio of composed 1:1 of sludge TS to pulp VS (Raposo *et al.* 2006; Liu *et al.* 2009), urea at a C:N ratio of 25:1 (Zhu 2007; Miqueleto *et al.* 2010), and adjusted with distilled water to 300 mL. The solution in each anaerobic bottle was saturated with nitrogen gas that was administered at a flow rate of 800 $mL{\cdot}min^{-1}$ for 5 min, which removed the oxygen from the solution and ensured that the solution in the bottle was in an anaerobic environment. The daily gas production was collected, and the biogas slurry was collected on days 1, 3, 5, 7, 11, 15, 19, 27, 35, and 43 and was analyzed. The pH value was measured until the gas production was mostly completed and the experiment was terminated.

Analytical Methods

pH measurement

The pH of the materials during the test was measured according to standard methods (National Environmental Protection Agency. 1989) and with a pH meter (Mettler Toledo Laboratory Instruments, Columbus, OH, USA). The pH meter was periodically calibrated with standard buffers at a pH of 3.00 and 6.86 to ensure measurement accuracy.

Determination of biogas production

The biogas production was measured using a saturated salt water method and measured every 24 h.

Anaerobic fermentation first-order kinetic model

The first-order kinetic model is used extensively to evaluate biogas production in anaerobic digestion of organic waste systems (Bilgili *et al.* 2009; Lei *et al.* 2010; Liang *et al.* 2011). In this study, it was assumed that the biogas produced from anaerobically digested plant fiber material under the influence of the Ca^{2+} concentration is in accordance with Eq. 1,

$$G = G_{\mathrm{T}}[1 - \mathrm{EXP}(-\mathrm{kt})] \quad (1)$$

$$\mathrm{In}[G_{\mathrm{T}}/(G_{\mathrm{T}} - G)] = \mathrm{kt} \quad (2)$$

where G is the biogas accumulation (cm^3) at time t (d), t is the time (d) over the digestion period, G_T is the biogas production potential (cm^3), and k is the first-order kinetic constant (d_1) (Lo *et al.* 2010).

Theoretical calculation of gas production

The theoretical gas production was estimated according to the Buswell and Mueller method based on the contents of cellulose ($(C_6H_{10}O_5)n$) and hemicellulose ($(C_5H_8O_4)n$) (Buswell and Mueller 1952). Thus, the following formulas were used to calculate the theoretical gas production of the fiber materials,

$$\text{Cellulose: } C_6H_{10}O_5 + H_2O \rightarrow 3CO_2\uparrow + 3CH_4\uparrow \quad (3)$$

$$G_1 = M \times x_1 \times 1000 \times 22.4 \times 6/162 \quad (4)$$

$$\text{Hemicellulose: } C_5H_8O_4 + H_2O \rightarrow 5/2CO_2\uparrow + 5/2CH_4\uparrow \quad (5)$$

$$\text{Total gas production: } G_2 = M \times x_2 \times 1000 \times 22.4 \times 5/132 \quad (6)$$

$$G_{TO} = G_1 + G_2 \quad (7)$$

$$\text{Conversion into 298.13 K: } G_T = 298.13\ G_{TO} / 273.13 \quad (8)$$

where M represents the mass of the fiber material (g), G_1 and G_2 are the theoretical gas production (cm^3) of cellulose and hemicellulose, respectively, the ideal gas volume is under the standard state conditions (cm^3), the cellulose and hemicellulose contents are x_1, x_2, the theoretical gas production is G_{TO}, the G_T is 298.13 cm^3, and the volume of 1 mol of the ideal gas under standard state conditions is 22.4 (m^3).

High-throughput sequencing

DNA from the anaerobic granular sludge was sequenced. Total DNA was extracted using an EZNA® soil kit (Omega Bio-tek, Norcross, US). The DNA concentration and purity were determined using a NanoDrop 2000 device. Agarose gel electrophoresis on a 1% gel was used to determine the quality of the extracted DNA. The V3-V4 variable region was amplified by polymerase chain reaction (PCR) using the primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). The PCR product was recovered using a 2% agarose gel, purified using an AxyPrep DNA Gel Extraction Kit instrument (Axygen Biosciences, Union City, USA), eluted with Tris-HCl, and then visualized with 2% agarose electrophoresis. Quantitative detection was performed using a QuantiFluorTM-ST (Promega, Madison, USA). The purified amplified fragments were constructed into a library of PE2*300 according to the standard protocol for the Illumina MiSeq platform (Illumina, San Diego, USA).

RESULTS AND DISCUSSION

Effect of the Ca^{2+} Concentration on the pH During Anaerobic Digestion

The ideal methanogenic pH is in a relatively narrow range (Ward *et al*. 2008), specifically 6.8 to 7.2. When the pH is too low, it will prolong the lag period of methane generation (Hao *et al*. 2012). The suitable pH value for hydrolysis and acidification is from 5.5 to 6.5 (Kim *et al*. 2003). The data shown in Fig. 1 reveal that with the progress of fermentation, the pH value of the fermentation broth in each anaerobic flask generally had an initial tendency to decrease and then to slightly increase. After 7 days of

fermentation, the pH of the fermentation broth in each anaerobic flask remained below 6.8, which was not in the ideal pH range for methane production. A possible reason for the decreased pH of the fermentation broth may be that volatile organic acids (VFA) were produced by the acidic bacteria during anaerobic fermentation, and this contributed to the acidity of the fermentation broth (Izumi *et al*. 2010). After 11 days of fermentation, the pH of the fermentation broth in each of the anaerobic bottles remained below 6.6, which would increase the lag of methane production at this lower pH range (Hao *et al*. 2012). However, the pH of the fermentation broth at this point is within the appropriate pH range for hydrolytic acidification. Noteworthy, starting from the second day, the pH values of the F, G and H were always lower than those of the other five test groups, which may be an important reason for the inferiority of the anaerobic digestion of these three samples compared to the other five samples. Accordingly, the high concentration of Ca^{2+} in the substrate (≥1,200 mg/L) led to the decrease of the pH of the fermentation broth during the fermentation, and the pH of the fermentation broth was maintained at a low level, which was not conducive to anaerobic fermentation, and the progress of methane generation process eventually led to the unsatisfactory anaerobic digestion of the granular sludge.

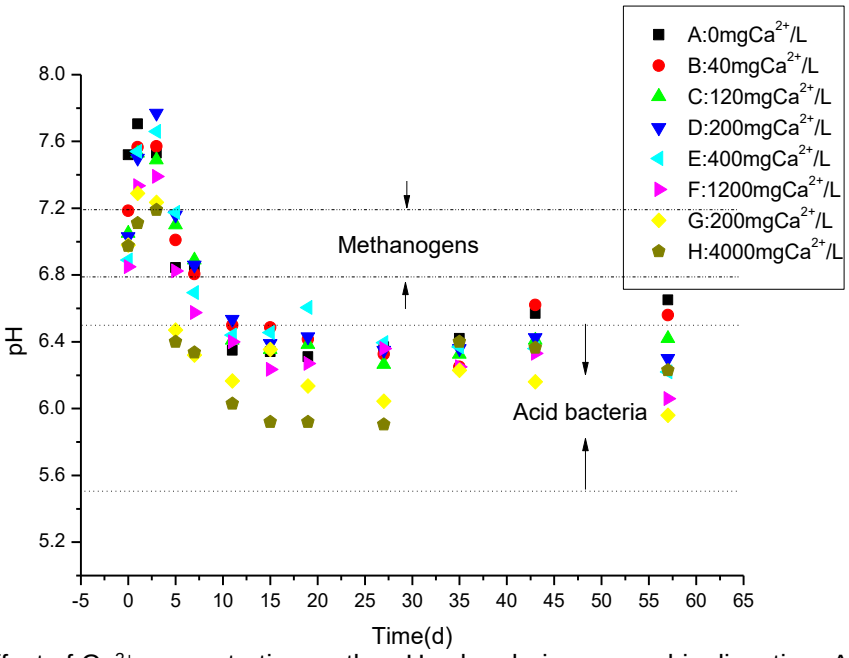

Fig. 1. Effect of Ca^{2+} concentration on the pH value during anaerobic digestion; A, B, C, D, E, F, G, and H represent the pH of the fermentation broth in the anaerobic bottles at 0, 40, 120, 200, 400, 1200, 2000, and 4000 mg/L Ca^{2+} concentrations, respectively

Effect of the Ca^{2+} Concentration on Anaerobic Digestion Cumulative Gas Production

The cumulative gas production from each sample is depicted in Fig. 2. Gas production by each sample started on day 3, and the cumulative gas production was the highest when the Ca^{2+} concentration in the substrate was 120 mg/L. In contrast, when the Ca^{2+} concentration in the substrate was 4,000 mg/L, the cumulative gas production was the lowest. Notably, when the Ca^{2+} concentration in the substrate was 40 mg/L (A) or 120 mg/L (B), the cumulative gas production was higher than that without Ca^{2+} addition. Also, when the Ca^{2+} concentration in the substrate was 200 mg/L (C), 400 mg/L (D),

1,200 mg/L (E), 2,000 mg/L (F), or 4,000 mg/L (H), the cumulative gas production was higher than that without Ca^{2+} addition. This data indicates that a low concentration of Ca^{2+} was beneficial to the anaerobic digestion of gas production from granular sludge, whereas a high concentration of Ca^{2+} inhibited the production of anaerobic digestion gas by granular sludge. The reason for this is that the low concentration of Ca^{2+} is conducive to the sludge granulation process, which increases the sludge activity and improves the ability of the sludge to degrade organic matter and the macroscopic performance is that the gas production from the anaerobic digestion of sludge is higher. The interaction between Ca^{2+} and granular sludge is actually the interaction of Ca^{2+} with the bacterial surface microenvironment. The high concentration of Ca^{2+} hinders the mass transfer efficiency of the material (Yang *et al*. 2010). Thus, the activity of the granular sludge is reduced, the ability of the sludge to decompose organic matter to produce gas is reduced, and the result in terms of macroscopic performance is that the production of anaerobic digestion of the sludge is low.

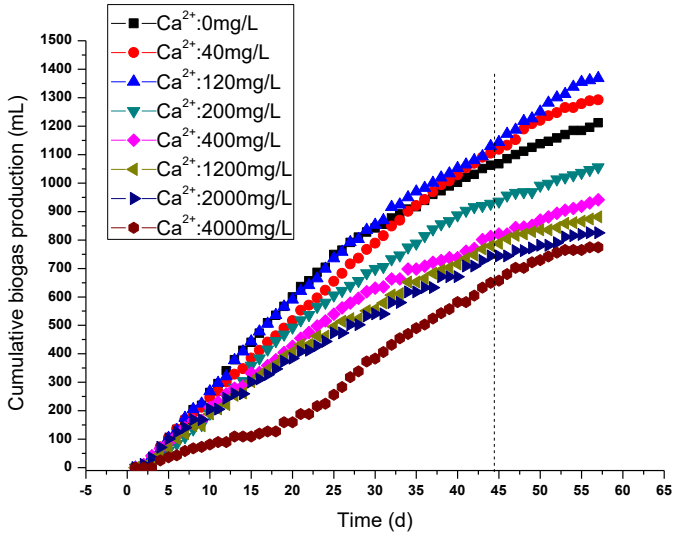

Fig. 2. Effect of Ca^{2+} concentration on gas production in anaerobic digestion; A, B, C, D, E, F, G, and H represent gas production in the anaerobic bottles at 0, 40, 120, 200, 400, 1200, 2000, and 4000 mg/L Ca^{2+}concentrations, respectively

Effect of the Ca^{2+} Concentration on the Anaerobic Digestion Gas Production Rate

The data shown in Fig. 3 indicate that with the increase of the Ca^{2+} concentration, the biogas generation rate initially increased and then decreased. During the entire anaerobic fermentation process, the final biogas production rate of each sample was as follows: 49.4, 52.7, 55.8, 43.0, 38.4, 35.9, 33.6, and 31.6% for samples A, B, C, D, E, F, G and H, respectively. Specifically, when the concentration of Ca^{2+} in the substrate was lower than 120 mg/L, the gas production rate of the anaerobic digestion of the fiber material increased with the increase of the Ca^{2+} concentration, which may have been attributable to the beneficial effect of low Ca^{2+} concentration on anaerobic fermentation to degrade the fiber material, indicating that Ca^{2+} promoted anaerobic fermentation. When the Ca^{2+} concentration in the substrate increased from 120 mg/L to 200 mg/L, the gas production rate of the anaerobic digestion of the fiber material rapidly decreased and was lower than that of the experimental group without Ca^{2+} addition. This finding

indicated that in this concentration range Ca^{2+} had an inhibitory effect on anaerobic fermentation. When the Ca^{2+} concentration in the substrate increased from 200 mg/L to 400 mg/L, the gas production rate of the anaerobic digestion of the fiber material also decreased rapidly and the gas production rate was obvious lower than that of the Ca^{2+} free group, which indicated that at this concentration, Ca^{2+} obvious inhibited anaerobic fermentation. When the concentration of Ca^{2+} in the substrate was higher than 400 mg/L, the effect of the Ca^{2+} concentration on the gas production rate of the anaerobic digestion of the fiber material was not clear. This may have been due to the tendency of the inhibition of anaerobic fermentation to be stable at such high concentrations of Ca^{2+}.

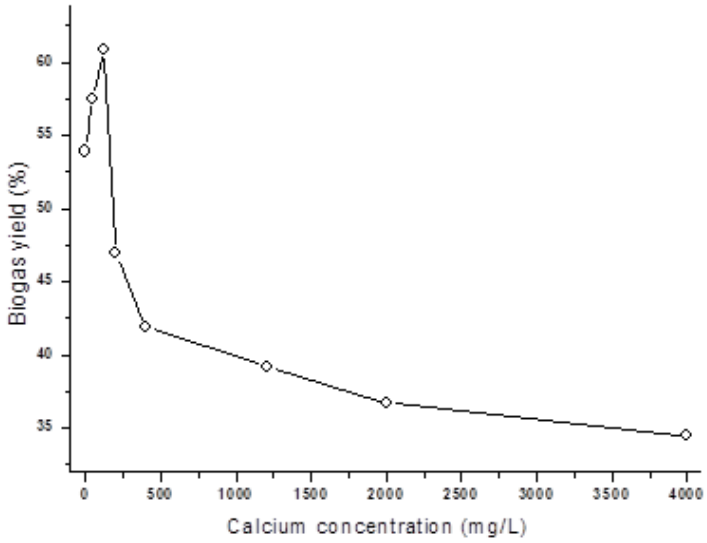

Fig. 3. Effect of the Ca^{2+} concentration on the gas production rate of anaerobic digestion

First-order Kinetic Simulation of the Effect of Ca^{2+} Concentration on the Cumulative Production of Biogas by Anaerobic Digestion

According to Eqs. 3 to 8, the theoretical gas production from each sample was 2453 cm^3, and then the first-generation biogas production rate constant *k* was obtained with Eqs. 1 and 2 as shown in Table 1. The cumulative gas production from each sample was consistent with the first-order kinetic model, and they all had a high coefficient of determination ($R^2 > 0.97$). Specifically, when the Ca^{2+} concentration in the substrate was 120 mg/L, the anaerobic digestion of the fiber material had the highest gas production rate. When the concentration of Ca^{2+} in the substrate was lower than 120 mg/L, the gas production rate of the anaerobic digestion of the fiber increased with increased Ca^{2+} concentration. However, when the concentration of Ca^{2+} in the substrate was ≥ 200 mg/L, the gas production rate of the anaerobic digestion of the fiber material decreased with the increase of Ca^{2+} concentration. Meanwhile, when the concentration of Ca^{2+} in the substrate was higher than 2,000 mg/L, the gas production rate of the anaerobic digestion of the fiber material was nearly the same. Therefore, it was concluded that the lower concentration of Ca^{2+} promoted the progress of the anaerobic decomposition of the fiber material, while the higher concentration of Ca^{2+} suppressed the progress of the anaerobic decomposition of the fiber material. Similarly, when the concentration of Ca^{2+} in the substrate was too high, the anaerobic fermentation was seriously affected. Accordingly, when using the anaerobically activated sludge process to treat wastewater containing lignocellulose, it was necessary to remove part of the water content as Ca^{2+}. In addition,

to ensure the anaerobic digestion, it was recommended that the concentration of Ca^{2+} in the water should not be higher than 200 mg/L.

Table 1. Effect of the Ca^{2+} Concentration on the Cumulative Biogas Production Rate by Anaerobic

Numbering	Calcium Concentration (mg/L)	k（d^{-1}）	R^2
A	0	0.01262	0.9853
B	40	0.01427	0.9981
C	120	0.01487	0.9987
D	200	0.01079	0.9870
E	400	0.00880	0.9892
F	1,200	0.00839	0.9907
G	2,000	0.00757	0.9899
H	4,000	0.00782	0.9743

Note: A, B, C, D, E, F, G, and H represent samples at 0, 40, 120, 200, 400, 1200, 2000, and 4000 mg/L Ca^{2+} concentrations, respectively

Granular Sludge Microbial Diversity Analysis

At the 97% classification level, alpha diversity is the analysis of species diversity in individual samples (Beazley *et al.* 2012). This mainly includes the Chao index, Ace index, Shannon index, Simpson index, and Coverage index. Chao or Ace is an index used to measure community abundance. The greater the index, the higher the degree of community richness. The Simpson index indicates the species diversity of the community. The smaller the Simpson index, the higher the species diversity. Coverage index represents the coverage of a single sample library, and the higher the Coverage index, the higher the probability that the sequence in the sample is measured.

As shown in Table 2, a total of 534,295 valid 16S rRNA sequences were obtained from the eight sludge samples, including 58643, 4755, 43084, 103897, 63167, 74249, 76505, and 67196 from samples A, B, C, D, E, F, G, and H, respectively. These gene sequences were divided into operational taxonomic units (OTU) at a 97%. Similarly, 567, 454, 549, 586, 631, 615, 455, and 552 were obtained from samples A, B, C, D, E, F, G, and H, respectively.

Table 2. Alpha Diversity Statistics of Anaerobic Granular Sludge

Sample ID	Reads				0.97		
		OTU	Ace	Chao1	Simpson	Shannon	Coverage
A	58643	575	623.51	623.62	0.1029	3.6462	0.9984
B	47554	423	549.08	544.39	0.0722	3.3566	0.9971
C	43084	553	609.26	608.38	0.0918	3.7753	0.9976
D	103897	596	641.87	663.00	0.0296	4.4143	0.9993
E	63167	690	725.96	737.35	0.0390	4.4913	0.9987
F	74249	714	763.92	769.78	0.0550	4.2299	0.9988
G	76505	450	515.25	521.03	0.0408	4.0701	0.9990
H	67196	552	609.09	623.40	0.1624	3.3415	0.9985

Note: A, B, C, D, E, F, G, and H represent granular sludge samples in anaerobic bottles at 0, 40, 120, 200, 400, 1200, 2000, and 4000 mg/L Ca^{2+} concentrations, respectively

Coverage of all sample libraries was greater than 99.7%, which indicated that the collected gene sequences represented the microbial communities of each sample well. The average Shannon index for each sample was 3.92, which was relatively high. This indicated that the number of microorganisms in each sample was relatively large. When the Ca^{2+} concentration in the substrate increased from 40 mg/L to 400 mg/L, the Shannon index increased from 3.36 to 4.49, and the diversity of microorganisms increased. When the concentration of Ca^{2+} in the substrate increased from 400 mg/L to 4000 mg/L, the Shannon index decreased from 4.49 to 3.34 and the diversity of microorganisms decreased.

None of the sample dilution curves (Fig. 4a) had a tendency to be saturated, indicating that additional diversity samples may not be detected by high-throughput sequencing (Zhang *et al*. 2012). The gradient of the grade abundance curve of each sample (Fig. 4b) was quite different suggesting that there is a great difference in species evenness (Sundberg *et al.* 2013) (B and G are less uniform). As the data in Fig. 4c shows, there were 226 identical OTUs in eight samples, accounting for 39.9 49.8, 41.2, 38.6, 35.8, 36.8, 49.7, and 40.9% of the OTUs in samples A, B, C, D, E, F, G, and H, respectively. The same number of OTUs accounted for less than 50% of the total number of OTUs for each sample. Thus, the Ca^{2+} substrate concentration of the sludge microbial community composition had a great impact.

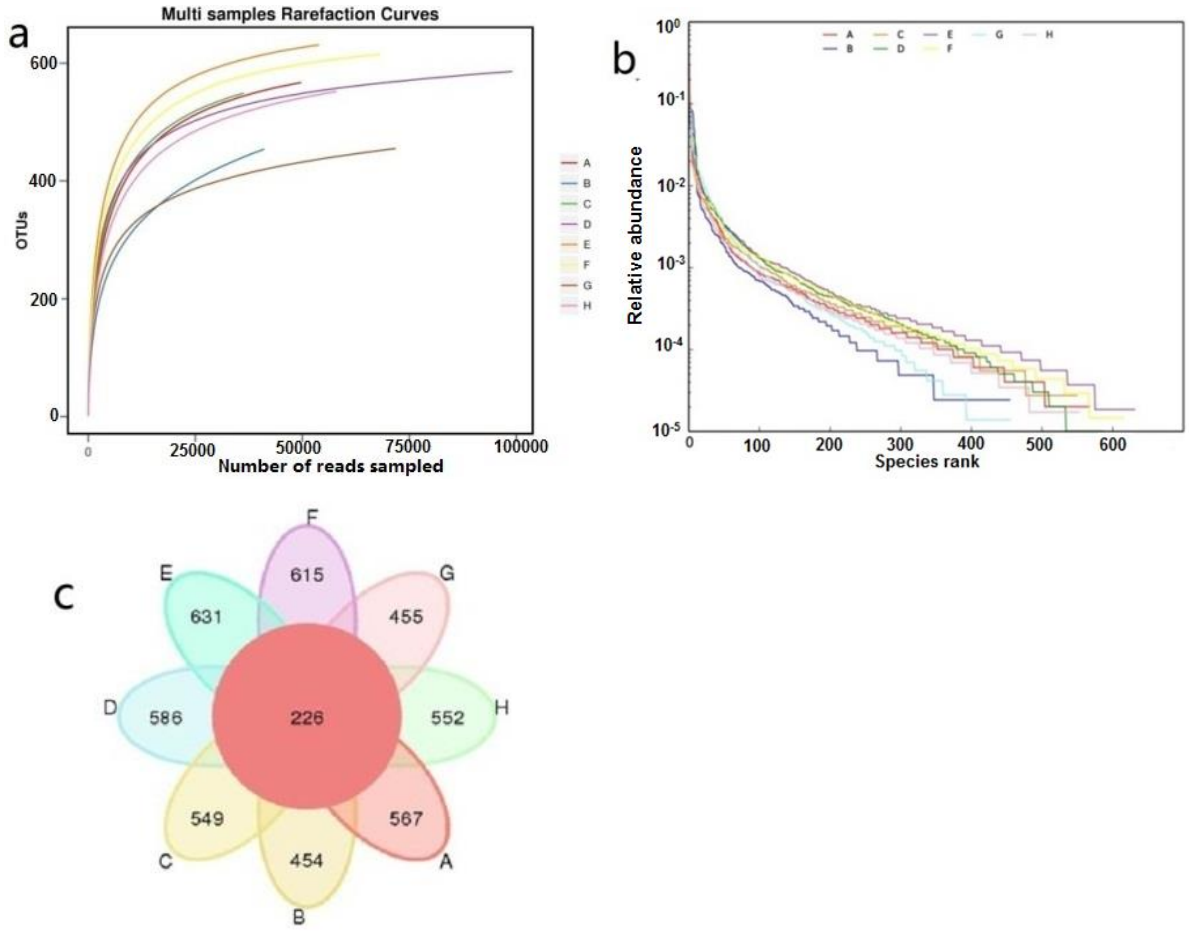

Fig. 4. a: Rarefaction curves; b: rank-abundance curves; c: Venn diagram of samples A, B, C, D, E, F, G, and H

Microbial Community Analysis

As shown in Fig. 5, as the concentration of Ca^{2+} in the substrate increased, the relative abundance of the phylum of bacteria *Firmicutes*, *Spirochaetae*, *Chloroflexi*, *Bacteroidetes*, and *Proteobacteria* was relatively high in all samples. These five bacteria were found to be biogas digesters and perform other anaerobic reactions of main bacteria in the phylum (Sundberg *et al.* 2013). The relative abundance in samples A, B, C, D, E, F, G, and H were 88.2, 81.2, 80.0, 70.8, 76.8, 61.6, 65.4, and 51.2%, respectively. With the increase of the Ca^{2+} concentration in the substrate, the relative abundance of these major bacterial generally showed a negative trend, and the difference in abundance was quite large. This indicated that the composition of sludge microorganisms was closely related to the concentration of Ca^{2+} in the substrate.

Firmicutes, which can secrete cellulase to hydrolyze acidified cellulose and hemicellulose, are an important acidified hydrolytic microorganism during anaerobic fermentation (Wen 2015). The relative abundance of *Firmicutes* increased with the Ca^{2+} concentration in the substrate when the Ca^{2+} concentration in the substrate was increased from 0 mg/L (in sample A) to 40 mg/L (in sample B). The relative abundance of *Firmicutes* decreased with the increase of the Ca^{2+} concentration, when the concentration of Ca^{2+} in the substrate increased from 40 mg/L (in sample B) to 2000 mg/L (kin sample H). It was speculated that the high concentration of Ca^{2+} led to the relative abundance of the phylum to reduce the bacteria level, which resulted in a slowdown of the acidification process in anaerobic digestion, and ultimately affected the entire process of anaerobic digestion. In addition, there is literature indicating that the stability of the anaerobic digestion process is positively correlated with the abundance of *Firmicutes* (Chen *et al.* 2016).

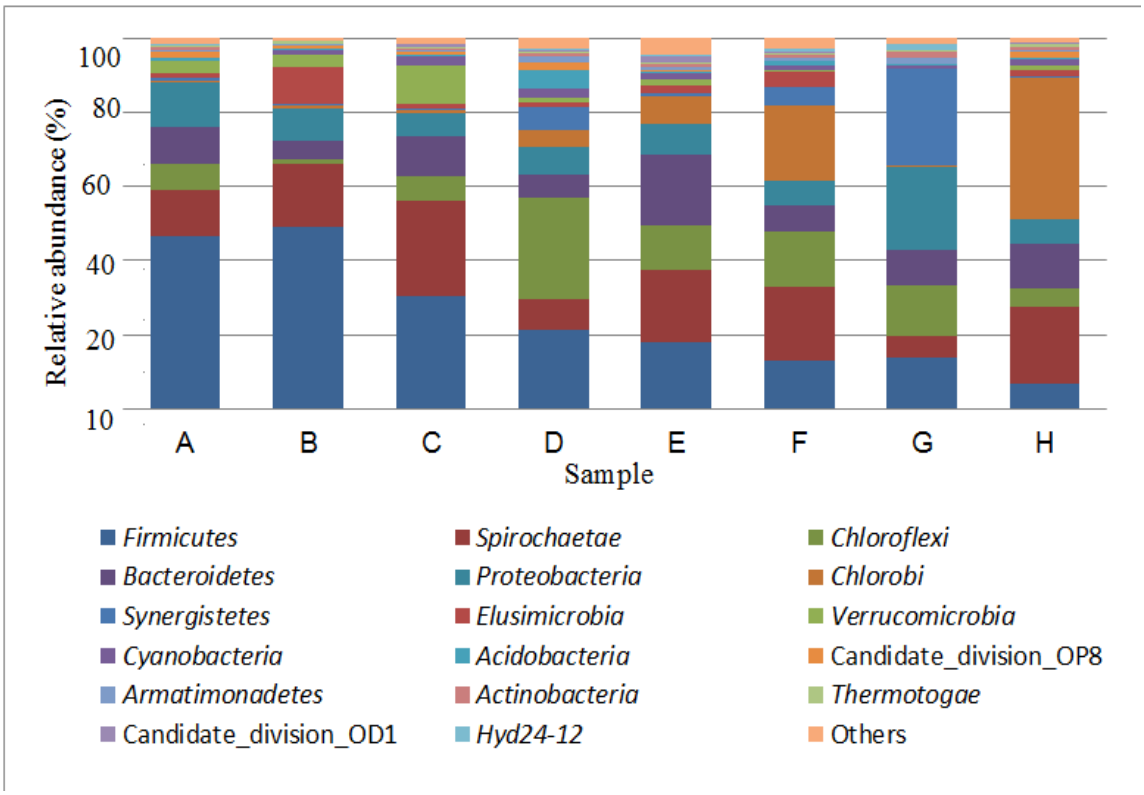

Fig. 5. Bacteria distribution at the phylum level in samples A, B, C, D, E, F, G, and H

As shown in Fig. 6, Ruminococcaceae members were relatively abundant in all samples. Ruminococcaceae belongs to the *Firmicutes* and degrades starch, cellulose, other polysaccharides, proteins, and short- and short-chain organic acids, and is an important bacterial family in the hydrolysis stage of anaerobic fermentation (Shen *et al.* 2013). The relative abundance of Ruminococcaceae decreased with increased concentration of Ca^{2+} in the substrate with lower Ca^{2+} concentrations (samples A and B). The relative abundance of Ruminococcaceae decreased with increased concentration of Ca^{2+} at higher concentrations of Ca^{2+} (samples D, E, F, G, and H). The decrease in the relative abundance of these species probably resulted in anaerobic sludge, which was one of the reasons of reduced digestion. Anaerolineaceae had the highest relative abundance of Ca^{2+} in the substrate at the concentration of 200 mg/L. When the concentration of Ca^{2+} in the substrate was higher than 200 mg/L, the relative abundance of these bacterial group gradually decreased with increased Ca^{2+} concentration. Available literature indicates that this family of bacteria has the ability to supply organic acids, such as acetates, to other microorganisms, such as acetic acid-producing methanogens (Liang *et al.* 2015). This may also be another important reason that leads to anaerobic digestion of the sludge.

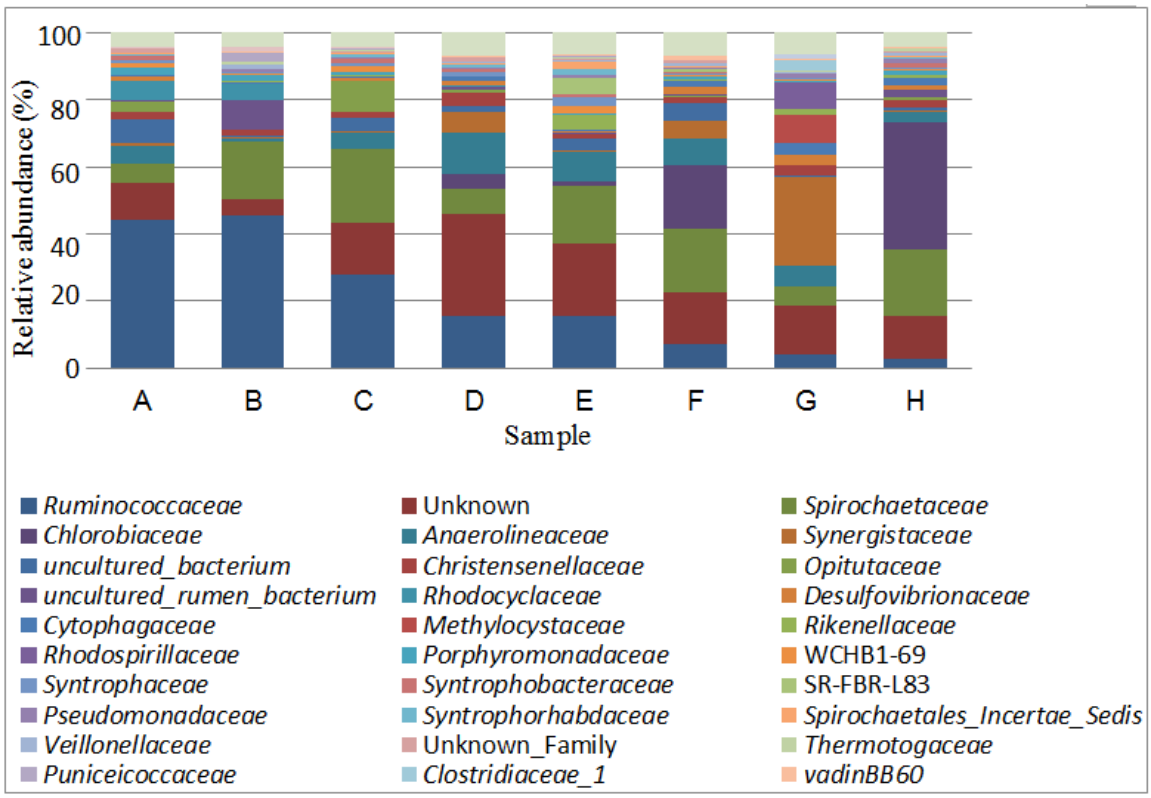

Fig. 6. Bacteria distribution at the family level in samples A, B, C, D, E, F, G and H

The interaction between Ca^{2+} and granular sludge is actually the interaction of Ca^{2+} with the bacterial surface microenvironment. Bacterial surfaces and bacterial extracellular polymers (EPS) are usually negatively charged, and their association with each other to form granular sludge requires cations, and Ca^{2+} as divalent cations strongly enhance the attachment of extracellular microbial appendages and extracellular polymers (Yu *et al.* 2001; Forster and Lewin 1972). However, the ability of EPS to bind Ca^{2+} is limited, and a large number of bindings lead to changes in the surface-active groups of

the EPS, thus affecting its original effect (Tourney and Ngwenya 2014). Changes in the content and nature of EPS will affect the microbial granulation process and thus influence the change of the microenvironment (Wang 2014). Accordingly, Ca^{2+} mainly reduces the biomass of granular sludge by blocking mass transfer channels, reducing nutrient elements and so on, and affects microbial communities by affecting the biomass.

CONCLUSIONS

1. Low Ca^{2+} concentration ($\leqslant$ 120 mg/L) increased the gas production rate and gas production efficiency of anaerobic fermentation. An excessively high Ca^{2+} concentration ($\geqslant$400 mg/L) resulted in anaerobic fermentation gas production. When the concentration of Ca^{2+} in the substrate was very high ($\geq$ 400 mg/L), the pH of the fermentation broth clearly decreased to a level that was lower than the ideal pH of anaerobic digestion.
2. When the concentration of Ca^{2+} in the substrate was higher than 40 mg/L, the Ruminococcaceae, an important bacterial family in the hydrolysis stage of anaerobic fermentation, sharply decreased. When the concentration of Ca^{2+} in the substrate was higher than 200 mg/L, the Anaerolineaceae, a family of bacteria capable of providing organic acids to other microorganisms such as the methanoic acid-splitting methanogens, was drastically decreased.

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REFERENCES CITED

Ahn, J. H., Do, T. H., Kim, S. D., and Hwang, S. K. (2006). "The effect of calcium on the anaerobic digestion treating swine wastewater,''*Biochemical Engineering Journal* 30(1), 33-38. DOI: 10.1016/j.bej.2006.01.014

Beazley, M. J., Martinez, R. J., Rajan, S., Powell, J., Piceno, Y. M., Tom, L. M., Andersen, G. L., Hazen, T. C., Van Nostrand, J. D., Zhou, J. Z., Mortazavi, B., and Sobecky, P. A. (2012). "Microbial community analysis of a coastal salt marsh affected by the Deepwater Horizon oil spill,''*Plos One* 7(7), e41305. DOI: 10.1371/journal.pone.0041305

Bilgili, M. S., Demir, A., and Varank, G. (2009). "Evaluation and modeling of biochemical methane potential (BMP) of landfilled solid waste: A pilot scale study," *Bioresource Technology* 100(21), 4976-4980. DOI: 10.1016/j.biortech.2009.05.012

Buswell, A. M., and Mueller, H. F. (1952). "Mechanism of methane fermentation," *Industrial and Engineering Chemistry Research* 44(3), 550-552. DOI: 10.1021/ie50507a033

Chen, S., Cheng, H., Wyckoff, K. N., and He, Q. (2016). "Linkages of firmicutes and bacteroidetes populations to methanogenic process performance," *Journal of Industrial Microbiology and Biotechnology* 43(6), 771-781. DOI: 10.1007/s10295-016-1760-8

Forster, C. F., and Lewin, D. C. (1972). "Polymer interactions at activated surfaces," *Effluent & Water Treatment Journal* 12(10), 520-525.

Han, F. B. (2008). *Research on Facilitated Formation Technology and Activity Conservation Method of Anaerobic Granules*, Ph.D. Dissertation, Northwest A&F University, Shaanxi, China.

Hao, L. P., Lü, F., Li, L., Shao, L. M., and He, P. J. (2012). "Shift of pathways during initiation of thermophilic methanogenesis at different initial pH," *Bioresource Technology* 126(12), 418-424. DOI: 10.1016/j.biortech.2011.12.072

Izumi, K., Okishio, Y., Nagao, N., Niwa, C., Yamamoto, S., and Toda, T. (2010). "Effects of particle size on anaerobic digestion of food waste," *International Biodeterioration & Biodegradation* 64(7), 601-608. DOI: 10.1016/j.ibiod.2010.06.013

Jackson-Moss, C. A., Duncan, J. R., and Cooper, D. R. (1989). "The effect of calcium on anaerobic digestion," *Biotechnology Letters* 11(3), 219-224. DOI: 10.1007/BF01026064

Kim, M., Gomec, C. Y., Ahn, Y., and Speece, R. E. (2003). "Hydrolysis and acidogenesis of particulate organic material in mesophilic and thermophilic anaerobic digestion," *Environmental Technology Letters* 24(9), 1183-1190. DOI: 10.1080/09593330309385659

Langerak, E. P. A., Gonzalez-Gil, G., Aelst, A., and Lettinga, G. (1998). "Effects of high calcium concentrations on the development of methanogenic sludge in upflow anaerobic sludge bed (UASB) reactors," *Water Research* 32(4), 1255-1263. DOI: 10.1016/S0043-1354(97)00335-7

Langerak, E. P. A., Ramaekers, H., Wiechers, J., and Lettinga, G. (2000). "Impact of location of $CaCO_3$ precipitation on the development of intact anaerobic sludge," *Water Research* 34(2), 437-446. DOI: 10.1016/S0043-1354(99)00154-2

Lei, Z., Chen, J., Zhang, Z., and Sugiura, N. (2010). "Methane production from rice straw with acclimated anaerobic sludge: Effect of phosphate supplementation," *Bioresource Technology* 101(12), 4343-4348. DOI: 10.1016/j.biortech.2010.01.083

Liang, B., Wang, L. Y., Mbadinga, S. M., Liu, J. F., Yang, S. Z., Gu, J. D., and Mu, B. M. (2015). "Anaerolineaceae and Methanosaeta turned to be the dominant microorganisms in alkanes-dependent methanogenic culture after long-term of incubation," *AMB Express* 5(1), 5-37. DOI: 10.1186/s13568-015-0117-4

Liang, Y., Zheng, Z., Hua, R., and Luo, X. (2011). "A preliminary study of simultaneous lime treatment and dry digestion of smooth cordgrass for biogas production," *The Chemical Engineering Journal* 174(1), 175-181. DOI: 10.1016/j.cej.2011.08.071

Liu, S. C. (2004). *Analysis and Detection of Pulping and Papermaking*, Chemical Industry Press.

Liu, G., Zhang, R., El-Mashad, H. M., and Dong, R. (2009). "Effect of feed to inoculum ratios on biogas yields of food and green wastes," *Bioresource Technology* 100(21), 5103-5108. DOI: 10.1016/j.biortech.2009.03.081

Lo, H. M., Kurniawan, T. A., Sillanpää, M. E., Pai, T. Y., Chiang, C. F., Chao, K. P., Liu, M. H., Chuang, S. H., Banks, C. J., Wang, S. C., *et al.* (2010). "Modeling biogas production from organic fraction of MSW co-digested with MSWI ashes in anaerobic

bioreactors," *Bioresource Technology* 101(16), 6329-6335. DOI: 10.1016/j.biortech.2010.03.048

Miqueleto, A. P., Dolosic, C. C., Pozzi, E., Foresti, E., and Zaiat, M. (2010). "Influence of carbon sources and C/N ratio on EPS production in anaerobic sequencing batch biofilm reactors for wastewater treatment," *Bioresource Technology* 101(4), 1324-1330. DOI: 10.1016/j.biortech.2009.09.026

National Environmental Protection Agency. (1989). *Water and Wastewater Monitoring Analysis Method*, China Environmental Science Press, Beijing, China.

Parkin, G. F., and Owen, W. F. (1986). "Fundamentals of anaerobic digestion of wastewater sludges," *Journal of Environmental Engineering* 112(5), 867-920. DOI: 10.1061/(ASCE)0733-9372(1986)112:5(867)

Raposo, F., Banks, C. J., Siegert, I., Heaven, S., and Borja, R. (2006). "Influence of inoculum to substrate ratio on the biochemical methane potential of maize in batch tests," *Process Biochemistry* 41(6), 1444-1450. DOI: 10.1016/j.procbio.2006.01.012

Shen, P., Zhang, J., Zhang, J., Jiang, C., Tang, X., and Li, J., Zhang, M., and Wu, B. (2013). "Changes in microbial community structure in two anaerobic systems to treat bagasse spraying wastewater with and without addition of molasses alcohol wastewater," *Bioresource Technology* 131(3), 333-340. DOI: 10.1016/j.biortech.2012.12.147

Speece, R. E. (1983). "Anaerobic biotechnology for industrial wastewater treatment," *Environmental Science & Technology* 17(9), 416A. DOI: 10.1021/es00115a725

Sundberg, C., Al-Soud, W. A., Larsson, M., Alm, M., Yekta, S. S., Svensson B. H., Sorensen, S. J., and Karlsson, A. (2013). "454 pyrosequencing analyses of bacterial and archaeal richness in 21 full-scale biogas digesters," *FEMS Microbiology Ecology* 85(3), 612-626. DOI: 10.1111/1574-6941.12148

Tourney, J., and Ngwenya, B. T. (2014). "The role of bacterial extracellular polymeric substances in geomicrobiology," *Chemical Geology* 386, 115-132. DOI: 10.1016/j.chemgeo.2014.08.011

Wen, B. T. (2015). *Studies on the Characteristics of Lignocellulose Degradation During the Process of Enzymatic Saccharification and Anaerobic Digestion*, Ph.D. Dissertation China Agricultural University, Beijing, China.

Wang, J. (2014). "Study on microbial community structure of nitrogen removal process in landfill bioreactor by using high-throughput sequencing and other molecular biological techniques," Ph.D. Dissertation, East China Normal University, Shanghai, China.

Ward, A. J., Hobbs, P. J., Holliman, P. J., and Jones, D. L. (2008). "Optimisation of the anaerobic digestion of agricultural resources," *Bioresource Technology* 99(17), 7928-7940. DOI: 10.1016/j.biortech.2008.02.044. Epub 2008 Apr 11.

Xue, F. Q., Sun, Z. L., Qian, M., Fu, H. X., and Zhao, X. F. (2015). "Study on reducing calcification risk of anaerobic granular sludge in waste water treatment in a recycled papermill," *China Pulp & Paper* 34(2), 19-24. DOI: 10.3969/j.issn.0254-508X.2015.02.005

Yang, S., He, Y., Liu, Y., Chou, C., Zhang, P., and Wang, D. (2010). "Effect of wastewater composition on the calcium carbonate precipitation in upflow anaerobic sludge blanket reactors," *Frontiers of Environmental Science & Engineering in China* 4(2), 142-149. DOI: 10.1007/s11783-010-0026-3

Yu, H. Q., Tay, J. H., and Fang. H. H. (2001). "The roles of calcium in sludge granulation during UASB reactor start-up," *Water Research* 35(4), 1052-1060. DOI: 10.1016/S0043-1354(00)00345-6

Zhang, T., Shao, M. F., and Ye, L. (2012). "454 pyrosequencing reveals bacterial diversity of activated sludge from 14 sewage treatment plants," *ISME Journal* 6(6), 1137-1147. DOI: 10.1038/ismej.2011.188

Zhu, N. (2007). "Effect of low initial C/N ratio on aerobic composting of swine manure with rice straw," *Bioresource Technology* 98(1), 9-13. DOI: 10.1016/j.biortech.2005.12.003

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