Pretreatment of piggery wastewater by a stable constructed microbial consortium for improving the methane production

Jian Cai, Xiwei Mo, Guojun Cheng and Dongyun Du

ABSTRACT

A stable aerobic microbial consortium, established by successive subcultivation, was employed to solubilize the solid organic fraction in swine wastewater. In the 30 days' successive biological pretreatments, 30–38% of volatile solids and 19–28% total solids in raw slurry were solubilized after 10 hours at 37 °C. Meanwhile, soluble chemical oxygen demand (COD) and volatile fatty acid increased by 48%–56% and 600%–750%, respectively. Furthermore, the molecular microbial profile of the consortium in successive pretreatment was conducted by denaturing gradient gel electrophoresis (DGGE). The results indicated that bacterial species of the consortium rapidly overgrew the indigenous microbial community of raw water, and showed a stable predominance at the long-term treatment. As a consequence of biological pretreatment, pretreatment shortened digestion time by 50% and increased biogas production by 45% compared to raw water in the anaerobic process. The microbial consortium constructed herein is a potential candidate consortium for biological pretreatment of swine wastewater to enhance biogas production.

Key words | biological pretreatment, DGGE, methane production, microbial consortium, swine wastewater

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INTRODUCTION

With the fast development of livestock husbandry, pig slurry is among the most abundant farm waste in China. Currently, anaerobic digestion is becoming the most attractive technology, which has been used by many concentrated animal feeding operations (Mitchell *et al.* 2013), since it produces methane, valuable digested residues, and liquid fertilizer (Chae *et al.* 2008). However, its low hydrolysis and biogas production rates can limit its economic feasibility. It is essential to find methods to improve the biogas potential of swine slurry.

Early studies reported the use of thermal hydrolysis and chemical pretreatment to increase the available soluble organic matter of swine manure prior to its anaerobic digestion. Researchers determined that heating the manure at 80 °C, or employing NaOH, was an efficient method to release the organic matter for methane productivity (Bonmati *et al.* 2001; González-Fernández *et al.* 2008). However, biological pretreatment has some advantages over physiochemical methods: no energy requirement, low operating costs, minimization of the secondary pollution risk, and high solubilization rates. Hemicellulose degrading bacterium exhibited a 30% increase of biogas production of manure through biological pretreatment (Angelidaki & Ahring 2000). The thermophilic aerobic bacteria were considered as a useful treatment to solubilize 20–30% of the organic sludge and increase 50% biogas yield (Hasegawa *et al.* 2000). In practice, the individual strain showed lower ability to degrade the complex waste. So the researchers constructed a microbial consortium with various degradability for recalcitrant matter such as lignocellulose (Wongwilaiwalin *et al.* 2010). Therefore, a new microbial consortium for biotreating the pig slurry is potentially a promising method to improve the biogas production.

In this paper, an aerobic microbial consortium was built to pretreat the pig slurry of a pig farm. The consortium was successively cultured from the aerobic batch reactors used to treat various farm wastewaters with high solid organics. The effects of the consortium in piggery sewage and subsequent methane production had been investigated. For industrial application, we focus our attention on the stability

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of the consortium and performance in the long-term pretreatment.

MATERIALS AND METHODS

Physiochemical characters of the pig wastewater

The raw swine wastewater was sampled from a pig farm with a yearly capacity of 40,000 fattening pigs in Huangshi city, Hubei province, China. As the slurry has been diluted with the wash water, the total solids (TS), volatile solids (VS), and chemical oxygen demand (COD) is much lower than raw manure. The characteristics of the samples are listed in the Supplementary Material (available online at http://www.iwaponline.com/wst/071/017.pdf).

Solid organic fraction degrading medium

A novel solid organic fraction degrading (SOFD) medium was designed to cultivate the microbial consortium. The solid fraction was separated from the pig slurry by 0.25 mm pore size screen and dried at 55 °C. The solution medium was composed of the following components: 5 g NaCl, 2 g $CaCO_3$, 4 g $(NH_4)_2SO_4$, 0.5 g $FeSO_4$, 20 g solid fraction described earlier, and 1 L H_2O.

Enrichment of the pretreating microbial consortium

Six sludge samples were collected from different plants, which are designed for treating farm waste (such as crop straw, swine manure, and dairy wastewater) in the countryside of Wuhan, China. Two grams of each sample were inoculated to the flask filled with 100 mL SOFD medium. These flasks were placed in a shaker at 37 °C at 100 rpm rotating speed for 3 days per cycle. At the end of each cycle, 95% of solution was removed, and the residue was used as seed sludge for the next cycle. After 10 repeated cycles, the six cultivated sludges were all mixed together as the microbial consortium. The microbial consortium was centrifuged at 5,000 rpm for 5 min, dried by freeze dryer to constant weight, and then powdered and stored at –20 °C. Particle size of the sludge powder was 0.57 μm.

Aerobic biological pretreatment batch experiments

Batch reactors were 2,000 mL flasks filled with pig slurry, tightly closed with silicone rubber stoppers and aluminum crimps, and then placed in a shaker at 37 °C at 100 rpm rotating speed for 20 hours. Every 2 hours, mixed slurry was sampled to analyze parameters: pH, volatile fatty acid (VFA), soluble COD (sCOD), VS, TS, and bacterial counts.

In the batch experiments, three treatments were carried out as follows: (P1) 1,000 mL fresh pig slurry and 1 g microbial consortium; (P2) 1,000 mL fresh slurry with 1 g sterilized consortium (sterilized at 120 °C for 1 hour); and (P3) 1,000 mL fresh slurry without inoculum as control group. Triplicates of these reactors were used in the experiment.

The successive pretreatment of piggery wastewater

To verify the stability of the pretreating performances by the microbial consortium, the consortium (1 g) was inoculated into the raw water (1,000 mL). Pretreatment was run in a 2,000 mL glass bottle with continuous stirring and a water jacket at 37 °C. The ventilation rate was at 0.4 vvm (volume per volume per minute). The untreated group was set to subtract the influence of raw slurry. After every 10-hours' treatment, 90% of the pretreated water was removed and analyzed, and the remainder was used as seed sludge for tackling new raw slurry. The whole successive pretreatment was carried out for 60 cycles during 30 days. As the repeated successive pretreatments were completely parallel repeated experiments in each cycle, we did not set the triplicate parallel experiment.

Determination of microbes and stability of the microbial consortium in pretreatment

Total bacterial DNA was extracted from the raw slurry consortium, and residual slurry of the successive aerobic reactors at the 1st, 10th, 20th, and 30th cycles, respectively. Each of the 2 g (fresh weight) samples was centrifuged at 8,000 rpm for 5 min, and next the supernatant was decanted. Genomic DNA was extracted according to the procedures described in the previous paper (Zhou *et al.* 1996). As reference (Li *et al.* 2010), a primer pair of 534R (5′-ATTACCGCGGCTGCTGG-3′) and 341F-GC (5′-CGCC CGCCGCGCGCGGCGGGCGGGGCGGGGGCCCTACG GGAGGCAGCAG-3′) was used to amplify the V3 region of 16S rRNA. The polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) procedure were operated as the reference (Leung & Topp 2001). Sequence data were aligned with the sequences of GenBank by the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

Anaerobic digestion experiment

The anaerobic inoculum (TS 348 g/L, VS 134 g/L) was obtained from a mesophilic fermentation reactor digesting pig slurry, stored at 4 °C. The last two cycles' pretreated slurry and untreated slurry were used to test the biogas improvement by biological pretreatment.

Digestion experiments were conducted in 2,000 mL glass vials, where 200 g of the anaerobic sludge was added into the glass bottle containing 1,500 mL pretreated slurry. Control reactors were set up in the same way for the untreated slurry, but 1.5 g of sterilized microbial consortium was injected into the control group. The reactors were maintained at 37 °C in a shaker and shaken several times per day to assure sufficient mixing. Triplicates of pretreated groups and controls were used in the experiment. The volume of methane production was measured automatically by water replacement methods (Zhang *et al.* 2011).

Analytical methods

These slurry samples were centrifuged at 7,000 rpm for 10 min and analyzed for sCOD. The sCOD, TS, VS, and VFA were determined according to the procedure listed in *Standards Methods for the Examination of Water and Wastewater* (APHA 2005). Microscope direct bacterial counts were performed as described previously (Leung & Topp 2001).

RESULTS AND DISCUSSION

The change of VFA, TS, VS, and sCOD in batch pretreatment

Firstly, for eliminating the extra organic matter from the inoculum, all the rates calculated herein had the background parameters subtracted from the increments of sterilized control: sCOD (274 mg/L), VFA (38.9 mg/L), TS (360 mg/L), and VS (110 mg/L). As illustrated in Figure 1, during the primary 10 hours, the accumulation of VFA reached the maximum concentration (1,544 mg/L) in the P1 group, but further pretreating led to a rapid drop of VFA (Figure 1(a)). This decrease may be due to a portion of VFA being utilized by the microbes. As reported, acetic acid and butyric acid concentrations of about 1,600 mg/L are optimum for the methanogenic bacteria (Wang *et al.* 2009). The pretreatment time was set at 10 hours to obtain an adequate yield of VFA.

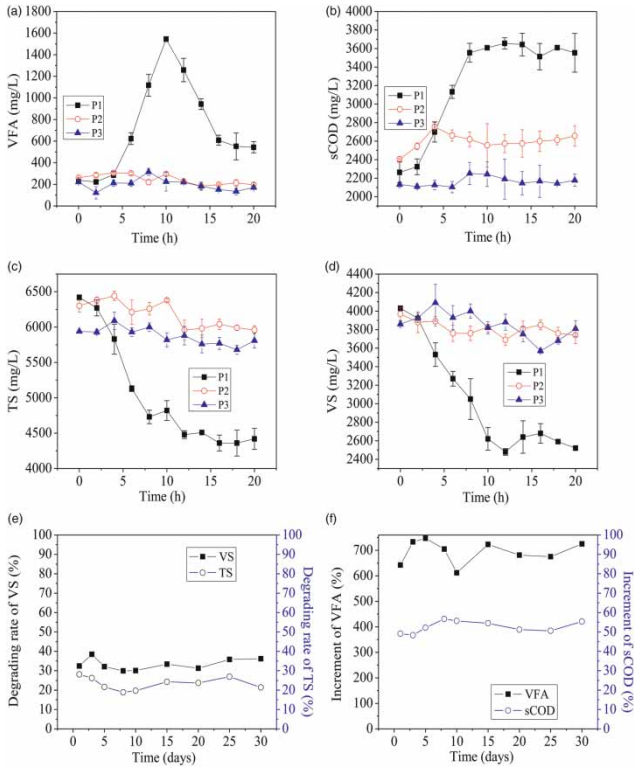

Figure 1 | Variations of VFA (a), sCOD (b), TS (c), and VS (d) of P1 (pretreated group), P2 (sterilized control), and P3 (raw slurry control) treatments in the batch pretreatment; and performances in the repeated successive experiment: (e) the removal rates of TS and VS and (f) the increments of VFA and sCOD.

The increasing of sCOD (Figure 1(b)) during the first 10 hours was in accordance with the VFA, but it did not show the same decreasing tendency as the VFA variation. This phenomenon may be due to the fact that sCOD do not only consist of VFA, the utilized VFA could also synthesize some other soluble organic matter. For example, the acetate of the VFA would be activated to acetyl phosphate by acetate kinase, which is subsequently converted to acetyl-CoA. As is well known, the acetyl-CoA is the substrate to synthesize fatty acid and other energy sources for microbes. And when the bacteria are growing, most bacteria produce extracellular polymeric substances, which could increase the sCOD (Laspidou & Rittmann 2002). The enhancement of sCOD was 47% in 10 hours, which was similar to the effect of adding alkali (González-Fernández *et al.* 2008). Corresponding to the sCOD, the removal rates of TS and VS were raised to a high level (17.7% and 34.3%, respectively) in 10 hours and stayed at a slightly fluctuating range. The decrease of VS and TS explained the increasing of sCOD. Briefly, the consortium is certainly useful for solubilizing the solid organics of swine slurry.

The stable effects in successive pretreatment

To observe the pretreating stability, several days' samples were randomly selected to show the tendency of operating performance. As shown, the increment of sCOD and VFA stayed at 46%–56% and 600%–750%, respectively (Figure 1(f)). Meanwhile, the removal rates of TS and VS stayed at 19%–25% and 30%–38%, respectively (Figure 1(e)). However, a better performance of 45% and 53% removal of TS and VS, respectively, was obtained on pretreating cow manure with NaOH (Li *et al.* 2009). The performance of the microbial consortium is basically reliable and effective, especially in the high production of VFA and sCOD.

Analysis of the main microbes in the consortium and pretreatment system

DGGE exhibits better reproducibility and reliability in the analysis of microbial diversity and succession of microbial community in the sludge than the traditional isolated culture methods (Sun *et al.* 2013).

Background smearing in the DGGE profiles indicated the microbial community in the raw water, microbial consortium, and pretreated system (Figure 2). Comparing the patterns of them, we observed that the indigenous microbes in pig slurry were basically replaced by the bacteria of the consortium. Except for bands G and H, which both presented in the consortium and raw slurry, the other five bands (A, B, C, D, and F) of original slurry completely disappeared in the pretreated water. Interestingly, these phylotypes were some species commonly found in the anaerobic environment (pig gastrointestinal tract or feces), notably *Lactobacillus*, Spirochaeta, *Bacteroides*, *Anaerobiospirillum*, and Flavobacteria. Under aerated treatment, these anaerobes were extremely inhibited and even eliminated. In addition, microbial populations enumerated by microscopic direct counts were about 1×10^7 cells/mL in the raw slurry. As the consortium was added, the bacterial population increased to 9.3×10^7 immediately, and finally increased to 1.3×10^9 cells/mL after 10 hours. The much higher content of the consortium was also an advantage to overwhelm the indigenous community.

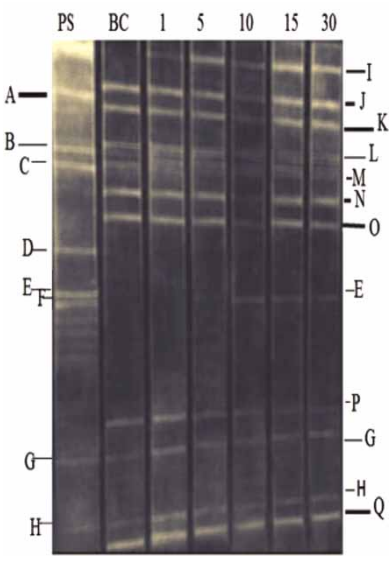

Figure 2 | DGGE patterns of the 16S rDNA bands of the microbial consortium and the microbial community in the successive pretreatments (PS lane, pig slurry control; BC lane, microbial consortium; 1–30 lane, the different pretreating time (days)).

In contrast to the bacteria of pig slurry, the consortium exhibited high stability during the successive pretreatment. Five most distinct bands were obvious in the lanes and no changes the profile. In this study, the five dominant bands, identified as *Bacillus amyloliquefaciens* (band J), *Bacillus cereus* (band K), *Pseudomonas* sp. (band N), *Paenibacillus* sp. (band O), and uncultured *Actinobacteria* bacterium, were all related to the aerobic species. *Bacillus* spp. are Gram-positive aerobes of the bacilli group, which have reported cellulase and xylanase production capacity (Okeke & Lu 2011; Wang *et al.* 2011). *Pseudomonas* sp. is active in degrading cellulose, lipids, sugars, and amino acids, and also commonly reported in cellulose-decomposing consortiums (Kinegam *et al.* 2007; Dumova & Kruglov 2009). *Paenibacillus* sp., facultatively aerobic spore-former, can grow at the expense of complex carbon sources such as whole rice, oats, or wheat, and can produce amylolytic, lipolytic, and proteolytic enzymes, suggesting that they could utilize solid organics of the swine slurry (Leung & Topp 2001; Liu *et al.* 2010). According to the reports, the ability of Actinobacteria to degrade starch, cellulose, and lignin is also undoubted (Kausar *et al.* 2011). These bacteria were possibly the predominant solid organic degraders, especially for lignocellulose.

However, several bands of consortium (L, M, P, G, and H) showed less intensity during the incubation. The bands (L and M), identified as uncultured bacterium and *Stenotrophomonas maltophilia*, are the members of the Gammaproteobacteria–Xanthomonadales–Stenotrophomonas group capable of aerobic growth on a variety of carbohydrates, and commonly produce the protease (Kornił-łowicz-Kowalska & Bohacz 2011). B and P were also the members of the Actinobacteria phylum. The bands G and H, identified as the species of clostridia and bacilli, were presented both in consortium and raw slurry, and were

Table 1 | Identities of the main bands of DGGE

Band	Accession number	Most similarity	Identity %	Classification
A	KP004207	Uncultured bacterium	98	*Flavobacteria*
B	KP004208	Uncultured bacterium	99	*Spirochaeta*
C	KP004209	Uncultured rumen bacterium	98	*Bacteroidia*
D	KP004210	Uncultured bacterium	96	*Gammaproteobacteria*
E	KP004211	*Alcaligenes* sp.	99	*Betaproteobacteria*
F	KP004212	Uncultured bacterium	99	*Bacilli*
G	KP004221	Uncultured bacterium	100	*Clostridia*
H	KP004222	*Bacillus subtilis*	99	*Bacilli*
I	KP004213	*Bacillus cereus*	99	*Bacilli*
J	KP004214	*Bacillus amyloliquefaciens*	100	*Bacilli*
K	KP004215	*Bacillus cereus*	100	*Bacilli*
L	KP004216	Uncultured bacterium	100	*Gammaproteobacteria*
M	KP004217	*Stenotrophomonas maltophilia*	100	*Gammaproteobacteria*
N	KP004218	*Pseudomonas* sp.	100	*Gammaproteobacteria*
O	KP004219	*Paenibacillus* sp.	100	*Bacilli*
P	KP004220	*Acinetobacter* sp.	100	*Gammaproteobacteria*
Q	KP004223	Uncultured bacterium	100	*Actinobacteria*

frequently found in the feces and urine. Clostridia are active in the manure or intestinal tract, fermenting the organic matter to produce VFA (Snell-Castro *et al.* 2005). But under aeration, their activity will be inhibited. However, the weak bands also showed degradability of some solid organic matter. Considering the diversity between the bacteria species, the pig slurry may not be the most appropriate substrate for them.

Despite the initial bacteria of the consortium, the novel band (I) appeared and exhibited a high increase in the pretreated water. B and E were derived from the pig slurry, disappeared in the prior 10 days, but were observed in the last 20 days in pretreatment. Based on the DNA analysis (Table 1), these two bands were identified as *Bacillus cereus* and *Alcaligenes* sp., which were reported as the dominant bacteria of cellulolytic and xylanolytic bacterial consortiums (Okeke & Lu 2011). These new bands indicated that the pretreatment environment was becoming more suitable for these cellulolytic phylotypes.

Overall, the identified members of the consortium and pretreatment belong mostly to the Bacilli, *Gammaproteobacteria*, and Actinobacteria. These types of bacteria are common in rice straw compost (Matsuyama *et al.* 2007) and rumen (Tajima *et al.* 1999), which suggest the importance of these types of bacteria for the degradation of solid organic materials in pig slurry.

Anaerobic digestion of the pretreated and un-pretreated wastewater

Two types of swine wastewater were used to test if the pretreatment increases biogas production. As illustrated in Figure 3(a), the pretreated slurry had significantly shortened 3 days after the start-up period compared to the raw water group. Similarly, 28% reduction of digestion time was also

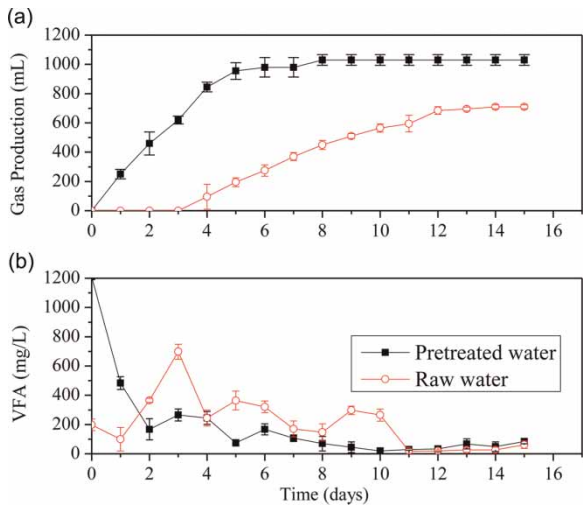

Figure 3 | Evolution of biogas yield (a) and the VFA concentration (b) in the anaerobic digestion.

obtained in a prior paper when they pretreated the primary sludge (Zheng *et al.* 2009). According to the VFA changes tendency (Figure 3(b)), the higher initial concentrations of VFA (1,200 mg/L) possibly promoted the biogas production of anaerobes. In the un-pretreated water, the biogas began to be produced with a longer lasting lag phase to accumulate the VFA at a sufficient concentration (about 700 mg/L). During the whole digestion, the gross biogas yield (1,030 mL) was much higher than the control group (710 mL). These results are in agreement with those published reports (Bonmati *et al.* 2001). They observed an increase of 45% in methane production, which equaled the one obtained in this study (45%). These results indicated that pretreated water was much more easily hydrolyzed and utilized by anaerobes.

CONCLUSIONS

We constructed an aerobic microbial consortium for solubilizing the organic solid in the pig slurry, and proved its stability and effectiveness as a pretreatment for anaerobic digestion. In the successive pretreatments, 30–38% of VS and 19–28% TS in the raw slurry were steadily solubilized. As a result, sCOD and VFA were increased 48%–56% and 600%–750%, respectively. PCR–DGGE results also proved the stability of the aerobic microbial consortium at a biological level. And DNA sequences showed that the most active genera in the pretreatment reactor were Bacilli, Gammaproteobacteria, and Actinobacteria, which were reported to have high degradability of cellulose and starch. When the pretreated slurry was used for fermentation, the amount of gas generated was increased by 45% compared to the untreated slurry. In addition, a shorter time for the starting period and 50% digestion time reduction was also observed. The present results demonstrate that the stable constructed microbial consortium has promising potential to improve biogas production.

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