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RESEARCH ARTICLE

Roles of acid-producing bacteria in anaerobic digestion of waste activated sludge

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HIGHLIGHTS

- Three acid-producing strains were isolated and identified.
- The isolated bacteria accelerated the anaerobic digestion processes.
- *Bacillus coagulans* improved TCOD removal, VS removal and biogas production.
- The optimal inoculum concentration of *Bacillus coagulans* AFB-1 was 30%.

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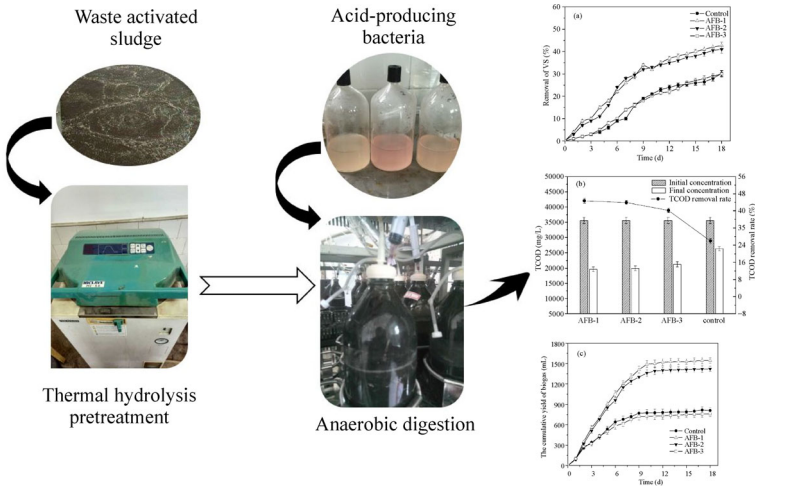
GRAPHIC ABSTRACT



ABSTRACT

Three acid-producing strains, AFB-1, AFB-2 and AFB-3, were isolated during this study, and their roles in anaerobic digestion of waste activated sludge (WAS) were evaluated. Data of 16S rRNA method showed that AFB-1 and AFB-2 were *Bacillus coagulans*, and AFB-3 was *Escherichia coli*. The removal in terms of volatile solids (VS) and total chemical oxygen demand (TCOD) was maximized at 42.7% and 44.7% by inoculating *Bacillus coagulans* AFB-1. Besides, the optimal inoculum concentration of *Bacillus coagulans* AFB-1 was 30% (v/v). Solubilization degree experiments indicated that solubilization ratios (SR) of WAS reached 20.8%±2.2%, 17.7%±1.48%, and 11.1%±1.53%. Volatile fatty acids (VFAs) concentrations and compositions were also explored with a gas chromatograph. The results showed that VFAs improved by 98.5%, 53.0% and 11.6% than those of the control, respectively. Biochemical methane potential (BMP) experiments revealed that biogas production increased by 90.7% and 75.3% when inoculating with *Bacillus coagulans* AFB-1 and AFB-2. These results confirmed that the isolated acid-producing bacteria, especially *Bacillus coagulans,* was a good candidate for anaerobic digestion of WAS.

1 Introduction

With the development of biological wastewater treatment, waste activated sludge (WAS) is an abundant byproduct every year. In China, the production of WAS was approximately 30 million metric tons in 2012, and it increased to 34 million metric tons in 2015 (Feng et al., 2015). Huge volumes of WAS must be treated rationally to reduce the potential environmental risks (Yang et al., 2010a; He et al., 2017). Biological treatment for pollution control is superior to physicochemical methods (Yang

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et al., 2010b; Cheng et al., 2016). Many attempts have been made to treat WAS through anaerobic digestion for biogas production. However, unlike hydrolytic acidification stage of simple substrates, WAS contains high-strength organic macromolecules (proteins and polysaccharides) whose decomposition is quite slow, and thus, it will result in the low fermentation rate for the anaerobic digestion of WAS. Yang et al. (2017) have also reported that H^+ has low oxidative-reductive potential (E_{H^+/H_2} = −414 mV, pH = 7.0) and it has difficulty in oxidizing most intermediates of anaerobic digestion at standard temperature and pressure. Under these conditions, the combination of thermal hydrolysis + anaerobic digestion is the most widely applied method at an industrial scale. During this process, WAS was hydrolyzed under a designated high temperature (>130°C) and pressure in a thermal hydrolysis reactor. In addition, a process included thermophilic anaerobic digestion by the microbial flora of the digestion tank, causing the organic matter to decompose and enhancing biogas production. Finally, the residual sludge was treated by dehydration and desiccation (Kepp et al., 2000).

Although the thermal hydrolysis pretreatment reportedly improved the hydrolysis efficiency, the biodegradability of WAS under anaerobic digestion was decreased with the increased temperature (Dwyer et al., 2008). Besides, once the temperature exceeds 121°C during the thermal hydrolysis procedure, most of the original microbes in the WAS will be killed, which works against the subsequent anaerobic decomposition of sludge. In addition, most microbial flora can not survive the thermophilic anaerobic digestion process, and the activity of microorganisms was restricted. The application of microbes to treat WAS does not result in additional environmental hazards based on the use of the bioremediation principle (Li et al., 2018). Therefore, it is necessary to screen and inoculate thermophilic strains into WAS to enhance the sludge decomposition and fermentation efficiency.

Acid-producing bacteria are the original microbes of WAS, and they are present in large amounts. Bohn et al. (2017) have found that a wide range of lactic acid-producing bacteria were present in biogas plants. It was previously shown that acid-producing bacteria could be isolated from thermophilic biogas plants (Cibis et al., 2016). Therefore, acid-producing bacteria are more likely to be screened out and cultivated under thermophilic anaerobic digestion than other microbes. The addition of acid-producing bacteria is performed on the basis of natural ecosystems. Thus, it would not lead to secondary pollution. In addition, during the acidification process, the hydrolysis products are converted into VFAs, alcohols and other products by acid-producing bacteria. In turn, these products are further degraded into methanogenic substrates (hydrogen, carbon dioxide and acetic acid). Schlüter et al. (2008) have identified some acid-producing bacteria as biogas-producing microbial communities. However, the research to date about the potential role of acid-producing bacteria in anaerobic digestion of WAS is limited.

Through this study, three acid-producing strains were isolated and added to the anaerobic WAS digester to improve the sludge decomposition and biogas production. The aim of this study was to investigate the impact of acid-producing bacteria on anaerobic digestion of WAS. In addition, experiments were performed to examine the optimal strain. The findings in this work might provide a cost-effective way to accelerate a reduction in the amount of WAS and improve its fermentation rate.

2 Materials and methods

2.1 Isolation of acid-producing bacteria

To screen the proper acid-producing strains, WAS samples were collected from a secondary sedimentation tank in a local wastewater treatment plant (Hunan, China). All samples were collected in sterile anaerobic bottles.

2.1.1 Culture medium

Liquid selective mediums (Cibis et al., 2016) were prepared for isolating acid-producing bacteria using the following compounds: glucose 8.0 g, peptone 1.5 g, K_2HPO_4 0.4 g, $(NH_4)_2SO_4$ 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.05 g, $FeCl_3$ 0.01 g, $MgCl_2$ 0.1 g, $CaCl_2$ 0.1 g, yeast extract 1.5 g, NaCl 3.0 g, L-cysteine 0.5 g, distilled water 1.0 L, and 0.1% methyl red indicator. The initial pH value was adjusted to 6.8 by adding HCl or NaOH. Solid culture mediums were produced by adding 1.7% (w/v) agar to the liquid selective mediums.

2.1.2 Isolation of acid-producing strains

The procedures for isolating the acid-producing strains were as follows:

1) Forty milliliters of WAS sample was added to 80 mL of liquid selective medium and cultured for 7 days. According to the literature (Cibis et al., 2016), the red culture mixture was selected as isolated acid-producing bacteria and diluted in a serial dilution (10^{-1}–10^{-7}) to obtain pure cultures.

2) The selected 0.2 mL of culture mixture from Step 1) was taken up with a sterile syringe and then inoculated onto solid mediums. After 3 days, the red colonies were selected, and purification was performed continuously 3–4 times until a single colony was formed and the medium colonies were consistent with the morphological features under an optical microscope. To obtain pure cultures, the streak plate technique was applied (Cibis et al., 2016). The three strains AFB-1, AFB-2 and AFB-3 were selected and preserved in a refrigerator at 4°C.

Anaerobic culture vessels were sealed and flushed with N_2 (99.99%) to maintain anaerobic conditions. All operations were performed under sterile conditions. The cultures were incubated at 50°C±0.3°C.

2.1.3 Bacterial identification

To identify the isolated bacteria, 16S rRNA genes were amplified by using a polymerase chain reaction (PCR) method. First, the total genomic DNA was extracted with a bacterial Genomic DNA Rapid Extraction kit (Bio Teke Corporation, Beijing, China) according to the protocol described by the manufacturer. The absorbance ratios (A260/A280) were measured with a micro-ultraviolet spectrophotometer (2000, NanoDrop Inc., USA) to check the purity of the extracted genomic DNA. When the A260/A280 ratios of genomic DNA were between 1.8 and 2.0 (Sharma et al., 2012), the total genomic DNA was extracted and used for PCR amplification. PCRs were then performed in a T-Gradient thermocycler (Biometra Gmbh, Gottingen, Germany). The universal bacteria primer 1 (27f: 5′-AGGTTTGACMTGGCTCAG-3′) and primer 2 (1492R: 5′-AGGTTTGACMTGGCTCAG-3′) were used for PCR amplification described by the references (Zhou et al., 2018). Each PCR amplification was performed in a volume of 30 μL. The PCR thermal program started at 95°C for 5 min, with 35 cycles of denaturation at 95°C for 30 s, 55°C annealing for 30 s, and at 72°C for a 1 min extension. A final extension was performed at 72°C for 10 min and then held at 12°C. The PCR products were purified using the Gel PCR Clean-Up System (Applied Biosystems, Foster, CA, USA). The sequences of the DNA fragments were studied by 3730XL DNA Sequencer method (Applied Biosystems, Foster, CA, USA). The nucleotide sequences of 16S rRNA were compared using the BLAST search option in the NCBI nucleotide sequence database (http://www.ncbi.nlm.nih.gov/BLAST/). The phylogenetic tree was created based on the maximum likelihood method by using MEGA 7.0.

2.2 Thermal hydrolysis of WAS

The WAS samples used were obtained from the secondary sedimentation tank in a local wastewater treatment plant (Hunan, China). The collected samples were filtered by using a 1 × 1 mm nylon microfilter to remove the large particles. The WAS used as feed was taken from a sewage treatment plant (Hunan, China) and cultured at 50°C± 0.3°C.

Four liters of WAS were added to 2 stainless kettles. The kettles were placed in an autoclave and pressure cooker for thermal hydrolysis. The treatment conditions were 130°C–170°C for 1 h according to Qiao et al. (2011). After that, the WAS samples were taken out and cooled for the subsequent anaerobic digestion experiment. The characteristics of the WAS samples were as follows: pH 7.1±0.1, volatile solids (VS) 5.9%±0.1%, total solids (TS) 6.2%± 0.3%, total chemical oxygen demand (TCOD) 35.6± 0.4 mg/L, and soluble chemical oxygen demand (SCOD) 10.8±0.62 mg/L.

2.3 Anaerobic digestion

Four replicate anaerobic reactors were performed with the volume of 1.0 L. One reactor without added acid-producing bacteria served as a control group and the other three reactors were inoculated with three isolated acid-producing bacteria. The inoculation proportion of the test groups were shown in Table 1. The entire digestion reaction lasted for 18 days at 50°C±0.3°C with shaking for 30 s twice a day.

To analyze the effects of acid-producing strains on the sludge hydrolysis, the solubilization ratio (SR) of sludge based on the increase of SCOD was investigated.

The SR of the organic components was measured according to the following equation (Lizama et al., 2017).

$$\mathrm{SR} = \frac{\mathrm{SCOD_{final}} - \mathrm{SCOD_{initial}}}{\mathrm{TCOD_{initial}}} \times 100\%, \qquad (1)$$

where SR is the solubilization ratio (%), $\mathrm{SCOD_{finial}}$ is the SCOD concentration of digested sludge when inoculating with acid-producing strains (mg/L), $\mathrm{SCOD_{initial}}$ is the SCOD concentration of WAS after thermal hydrolysis pretreatment (mg/L), and $\mathrm{TCOD_{initial}}$ is the TCOD concentration of WAS after thermal hydrolysis pretreatment (mg/L).

To verify the role of acid-producing bacteria on acidification process, the VFAs concentrations and compositions in four experimental groups were investigated by using a gas chromatograph (Agilent 7890A, USA). In addition, biochemical methane potential (BMP) experiments were also employed to illuminate the effect of acid-producing strains on the process taking place in the anaerobic reactors. The BMP assays were performed in

Table 1 Different inoculation conditions at 50°C±0.3°C

Group	Content
Control	300 mL seed anaerobic sludge + 400 mL WAS after thermal hydrolysis pretreatment + 300 mL sterile ultrapure water
AFB-1	300 mL seed anaerobic sludge + 400 mL WAS after thermal hydrolysis pretreatment + 300 mL bacteria suspension of *Bacillus coagulans* AFB-1
AFB-2	300 mL seed anaerobic sludge + 400 mL WAS after thermal hydrolysis pretreatment + 300 mL bacteria suspension of *Bacillus coagulans* AFB-2
AFB-3	300 mL seed anaerobic sludge + 400 mL WAS after thermal hydrolysis pretreatment + 300 mL bacteria suspension of *Escherichia coli* AFB-3

anaerobic reactors and sealed with butyl-rubber stoppers. The substrate (seed anaerobic sludge and WAS after thermal hydrolysis pretreatment) and inoculum (bacteria suspension or sterile ultrapure water) were mixed and added to the 1.0 L serum bottles (Table 1). The headspaces of BMP bottles were sealed and flushed with N_2 (99.99%) for 1 min. The reactors lasted for 18 days at 50°C±0.3°C with shaking for 30 s.

2.4 Optimization experiments

For the optimization studies, the four levels of initial strain inoculation rates examined were 10%, 20%, 30% and 40% (v/v). The cell density was measured with a spectrophotometer (721, Hengping Co., Ltd., Shanghai, China). All experiments were performed in duplicates.

2.5 Analytical methods

The pH value was measured with a pH meter (PHS-3C, Jinpeng Co., Ltd., Shanghai, China), and VFAs were determined using a gas chromatograph (Agilent 7890A, USA) with a flame ionization detector (FID) and a DB-FFAP (Agilent, USA) column (30 m × 0.32 mm × 0.50 μm). The ammonia nitrogen (NH_4^+-N) concentrations were determined by Nessler's reagent colorimetric method. TS, VS, TCOD and SCOD were measured using corresponding standard methods described by the American Public Health Association (APHA, 2005) . SCOD was obtained by centrifuging the WAS sample (5000 r/min for 5 min) and filtering the supernatant through a glass fiber filter (AP40, Aiyan biotechnology Co., Ltd., USA). TCOD was measured by direct COD analysis. The biogas production during anaerobic digestion was collected through drainage device. The volumes of biogas yields were measured by the water displacement.

3 Results and discussion

3.1 Isolation and identification of acid-producing bacteria

After four repetitions by streaking colonies onto solid mediums, three representative acid-producing bacteria were isolated. The AFB-1 and AFB-2 colonies were round and non-transparent with wet surfaces, while the AFB-3 colony had a smooth surface and exhibited a small bulge in the center. The total sequence lengths of three strains were 1561, 1786 and 1496 bp, respectively. The 16S rRNA sequences amplified from strain AFB-1 (accession No. KY170856) and AFB-2 (accession No. KY170857) showed 99% similarity with *Bacillus coagulans* (KC886323.1). Strain AFB-3 (accession No. KY170858) shared 99% similarity with *Escherichia coli* (AB680199.1). Figure 1 presented a phylogenetic tree based on16S rRNA gene sequences of the isolated strains. Strains AFB-1 and AFB-2 clearly belong to *Bacillus coagulans*. Strain AFB-3 was *Escherichia coli*.

3.2 Effect of acid-producing bacteria on the anaerobic digestion of WAS

3.2.1 Variations in the NH_4^+-N and pH value

The variations in the NH_4^+-N concentrations and pH value over time in WAS for 18-day culture were measured, and the results were presented in Fig. 2.

The optimal NH_4^+-N concentration was the key to the stability of the digestion process. A high ammonia concentration could inhibit the microbial activity (Lv et al., 2016), which generally was the primary cause of anaerobic digestion failure. As shown in Fig. 2(a), the NH_4^+-N concentrations of all series exhibited the same trend through the anaerobic digestion process. The NH_4^+-N concentrations in all groups first rose during the initial anaerobic digestion process (up to the 3rd day) followed by a decline after the 3rd day, which was similar to other findings (Yu et al., 2017b). As the anaerobic digestion continued, the NH_4^+-N concentrations remained moderate. The final NH_4^+-N concentrations in the experimental groups were significantly lower than that of the control group. In addition, the maximum NH_4^+-N concentrations in groups AFB-1, AFB-2, and AFB-3 were 15.0±0.50 mg/L, 21.0±0.44 mg/L and 22±0.50 mg/L, respectively. This result did not have a negative effect on the process given that the nitrogen concentrations ranged from 15.0 to 25.0 mg/L, which indicated that the addition of acid-producing bacteria might not inhibit the subsequent anaerobic digestion process.

As shown in Fig. 2(b), the pH value in all cases dropped from 7.1 to 6.5 during the first 3 days, and the peak appeared on the 6th day. Nonetheless, pH slowly decreased to 6.0–7.0 over the following 9 days. According to reference (Hwang et al., 2004), the appropriate pH range for fermentative microorganism was between 4.0 and 8.5. Therefore, the microbes involved in anaerobic digestion could act in the experiment. In addition, the pH value trends in all trials were contrary to the VFAs content trends. As shown in Fig. 2(b), the lowest pH emerged on the 9th day and pH was 6.0, 6.79 and 7.19 in groups AFB-1, AFB-2 and AFB-3. Whereas the higher VFAs content was found at the same time (Fig.2(c)). A similar phenomenon was observed by Jiang et al. (2013). In addition, Babel et al. (2004) also reported that the best result for VFAs production was obtained at pH 7 and VFAs production was halved at pH higher than 7. Therefore, the addition of acid-producing bacteria had a beneficial effect on the anaerobic digestion process, especially for VFAs production. After the 9th day, pH value rose due to the consumption of VFAs by methanogenic bacteria and then

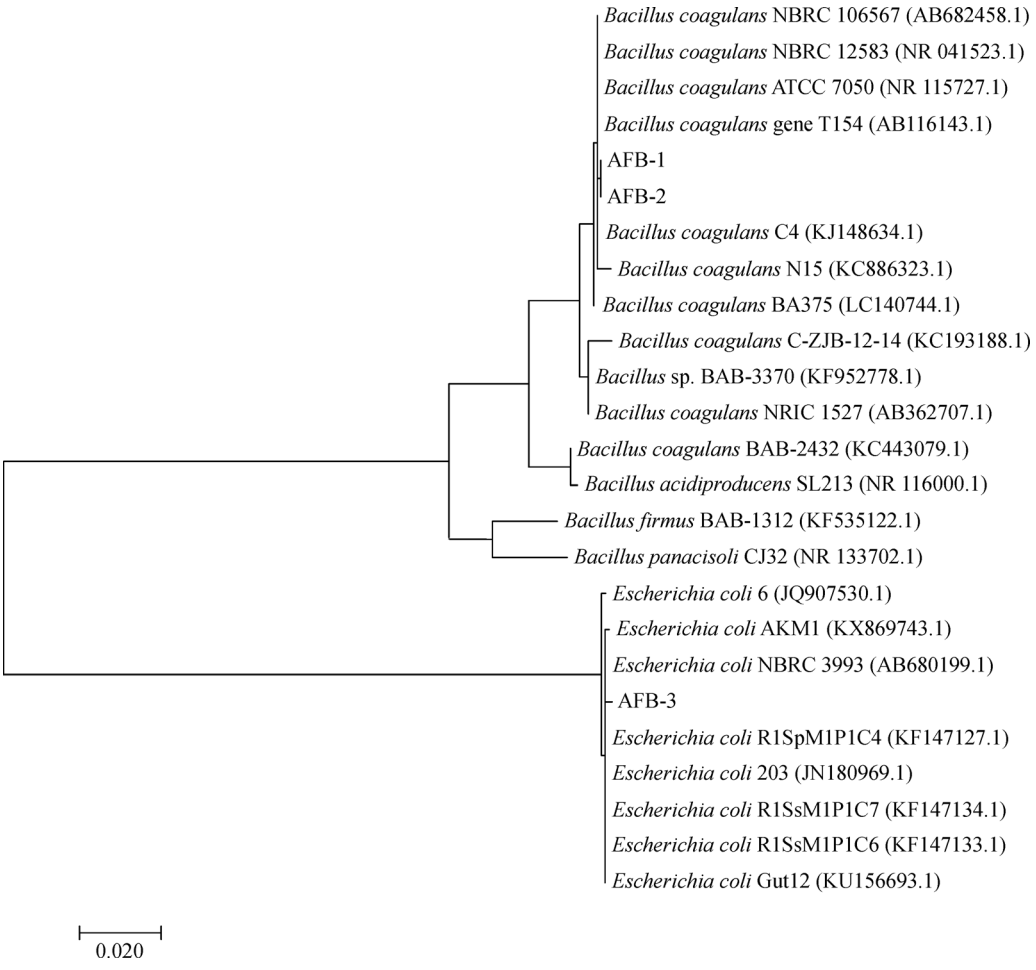

Fig. 1 Phylogenetic tree showing the relationships among 16S rRNA sequences from *Bacillus coagulans* AFB-1, *Bacillus coagulans* AFB-2 and *Escherichia coli* AFB-3 and the most similar sequences retrieved from databases

remained stable. The VFAs consumption and production reached a balance during anaerobic digestion.

3.2.2 VS and TCOD removal

VS removal was directly related to the reduction and stabilization of WAS. Thus, the removal of VS was studied to evaluate the anaerobic digestion performance by inoculating acid-producing bacteria. As shown in Fig. 3 (a), VS was gradually decreased with the increasing treatment time. The removal of VS after adding acid-producing bacteria was comparable to that of the control. On the 6th day, VS removal of groups AFB-1, AFB-2 and AFB-3 reached 22.1%±2.2%, 24%±3.1% and 10.2%± 1.5%, whereas the removal of the control was only 9%± 1.3%. These findings indicated that the addition of acid-producing bacteria enhanced the biodegradability of WAS. In addition, VS removal of groups AFB-1 and AFB-2 was higher than those found by Ding et al. (2017). This finding might be due to the different sources of sludge and the operation of experiments. In the end, VS removal of groups AFB-1, AFB-2 and AFB-3 reached 42.7%±1.3%, 41.0%±2.1% and 30.0%±1.6%, respectively. On one hand, the reduction of VS could be due to cell lysis under the thermophilic temperature and the release of plentiful organic components into the supernatant. On the other hand, a large proportion of soluble organic matter (SCOD) was converted by acid-producing bacteria (Yan et al., 2008). Moreover, the addition of acid-producing bacteria (strains AFB-1 and AFB-2) promoted the reduction of sludge by 12.5%±0.5% and 11.0%±1.3%, respectively, supporting the successful application of acid-producing bacteria in the domain of anaerobic digestion. In particular, Fig. 3(a) appeared that strain AFB-1 was the optimal strain in this experiment based on the degradation of VS.

The changes in TCOD were also explored during anaerobic digestion (Fig. 3(b)). The results showed that the TCOD concentrations in all series declined under anaerobic digestion, which was similar to the research by Ding et al. (2017). In addition, TCOD concentrations in the experimental groups decreased much faster than that of the control. Besides, TCOD removal rate (44.7%) of group

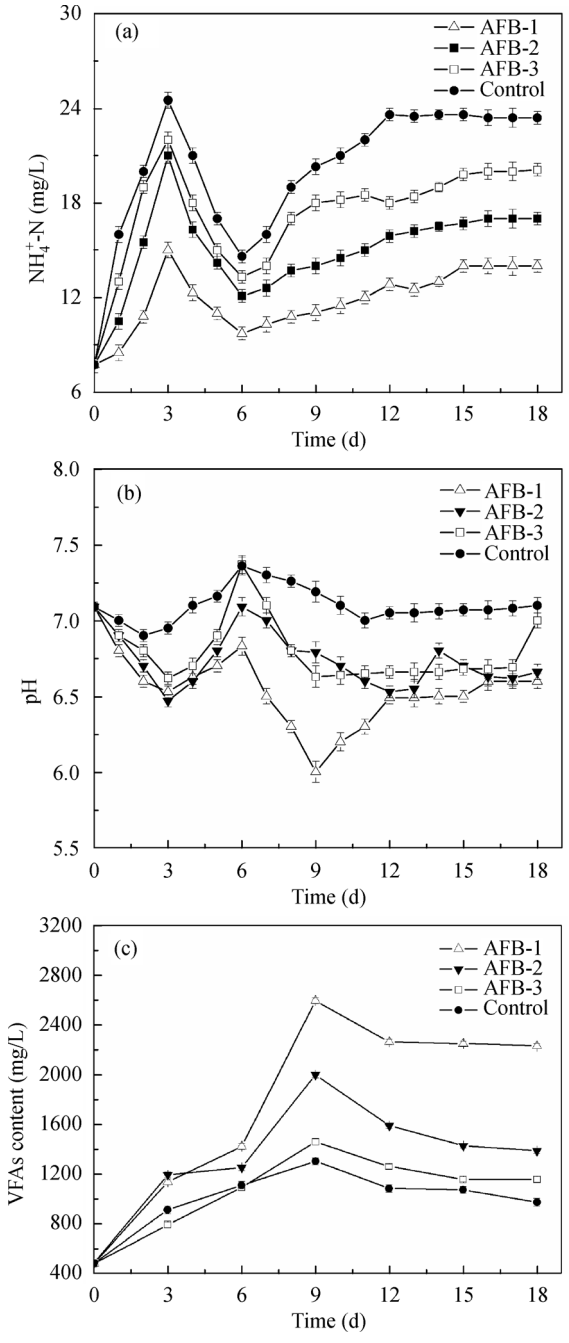

Fig. 2 Changes in the NH_4^+-N content, pH value and VFAs content with anaerobic digestion: (a) NH_4^+-N content; (b) pH value; and (c) VFAs content. Error bars represent standard deviations of triplicate tests

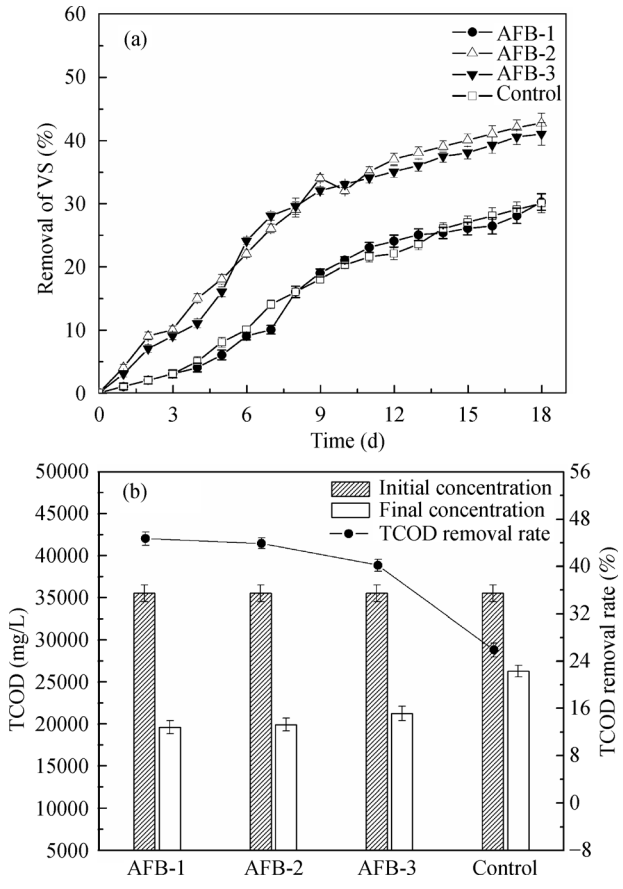

Fig. 3 Changes in VS removal and TCOD removal: (a) VS removal and (b) TCOD removal. The reaction temperature was 50°C±0.3°C. Error bars represent standard deviations of triplicate tests

AFB-1 was superior to that of group AFB-2 (43.9%) and group AFB-3 (40.2%). Thermostable enzymes could be released because less-temperature-tolerant microorganisms died, and the released thermostable enzymes could improve the degradation efficiency of WAS (Wu et al., 2017). Additionally, thermophilic microorganisms could achieve fast growth due to the utilization of released compounds. These findings showed that the removal of TCOD was a composite result of the degradation of organic compounds and the metabolic process.

3.3 Details of how acid-producing bacteria affect the anaerobic digestion performance of WAS

3.3.1 Effect of acid-producing bacteria on SCOD concentrations and SR of the organic components

The sludge compound degradation and microbial cell disruption resulted in the release of organic compounds in a soluble form (SCOD) into the supernatant. Although most of the organic compounds in WAS would be degraded and SCOD was released by thermal hydrolysis pretreatment, the solubilization of WAS would continue during the entire anaerobic digestion period and organic matter was continually solubilized to produce VFAs by interrelated microbial consortia (Liu et al., 2009). In addition, SCOD concentrations of WAS were related to the abundance of the microbial consortia (Liu et al., 2009). Thus, SCOD concentrations during the digestion and SR of the organic components were evaluated during anaerobic

digestion. According to Table 2, the SCOD concentrations in groups AFB-1, AFB-2, AFB-3 and the control increased from 10.8±0.62 g/L to 18.2±0.17 g/L, 17.1±0.32 g/L, 14.7±0.26 g/L and 14.5±0.23 g/L, respectively. This phenomenon was in accordance with (Kavitha et al., 2015). WAS involved plenty of organic components, and 60% of its components were proteins and carbohydrates (Guo et al., 2013; Peng et al., 2014). The remaining living microbes in the sludge would consume the released organic components and cause an increase in SCOD concentrations (Yu et al., 2017a), which demonstrated that more soluble proteins and soluble carbohydrates would be consumed by acidogenic microorganisms, and they were produced for acidification. These findings also illustrated that acid-producing strains accelerated the solubilization and cell destruction of the sludge. In particular, the maximum SR was 20.8%±2.20% when *Bacillus coagulans* AFB-1 was applied. This value was lower than the value (26%) reported by Lizama et al. (2017), whose experiment was conducted by ultrasonic pretreatment. The increase in the soluble concentration of organic compounds accelerated the metabolism of bacterial and archaeal communities in the subsequent phases. Consequently, the rate of biogas production was accelerated, and the biogas yields could be increased. The organic components could be exploited by fermentative bacteria. The solubilization of organic matter created a more biodegradable substrate for anaerobic microorganisms.

Table 2 Concentration and solubilization ratio of the organic components of digested sludge by inoculating acid-producing bacteria

Group	$SCOD_{initial}$ (g/L)	$SCOD_{final}$ (g/L)	$TCOD_{initial}$ (g/L)	Solubilization ratio (%)
Control	10.8±0.62	14.5±0.23	35.6±0.4	10.4±1.45
AFB-1		18.2±0.17		20.8±2.20
AFB-2		17.1±0.32		17.7±1.48
AFB-3		14.7±0.26		11.1±1.53

3.3.2 Effect of acid-producing bacteria on the VFAs concentrations and compositions

VFAs were intermediate compounds that were involved in the anaerobic digestion process of methane fermentation. Thus, the changes in VFAs (acetate, propionate, n-butyric acid, and isobutyric acid) over the 18-day experiment were investigated (Fig. 4). The VFAs concentrations in the experiments first increased and then decreased. Due to the conversion of soluble proteins and soluble proteins by acidogenic bacteria, VFAs concentrations increased to their peak. Subsequently, VFAs contents decreased to the minimum because methanogenic bacteria converted them for methane production. The trend in VFAs concentrations was consistent with Dearman and Bentham (2007).

It was also observed that the VFAs concentrations were positively affected by acid-producing bacteria during anaerobic fermentation. For example, during the first 3 days, the VFAs production showed the following trend: AFB-2>AFB-1>Control>AFB-3. After the 9th day, the highest VFAs production was obtained. The VFAs production of groups AFB-1, AFB-2 and AFB-3 were approximately 2594 mg/L, 1999 mg/L and 1458 mg/L, respectively. These concentrations improved by 98.5%, 53.0% and 11.6% compared with those obtained in the control group, respectively. On the 12th day, the process remained steady and the final VFA concentrations followed the pattern AFB-1>AFB-2>AFB-3>Control.

However, the accumulation of VFAs would inhibit the acidogenic process. Viéitez and Ghosh (1999) found that the acidogenic process of anaerobic digestion would be limited when VFAs concentrations exceeded 13000 mg/L. The higher VFAs concentrations would affect methane-producing activity and reduce methane production. As shown in Fig. 4, the VFAs concentrations in the experimental groups were below 13000 mg/L, which indicated that the addition of acid-producing bacteria to the anaerobic digestion of WAS led to no acid inhibition. When VFAs were consumed in large amounts, this consumption reflected the high activity of methanogens. Finally, VFAs were depleted in all the series, and the methane production no longer increased after the 12th day, indicating that the further decomposition of substrates into organic acids was limited.

The primary products of the acidogenic process were short chain fatty acids (C_1 to C_5). Acetic, butyric, propionic and formic acids were related to the fermentation of carbohydrates, proteins and lipids. The VFAs with higher molecular weights (valeric and isovaleric acids) were related to the fermentation of proteins. Figure 4 showed the variations in individual VFA. The major acid was found to be acetic acid. A similar phenomenon was observed by Zhang et al. (2017), who applied ZnO engineered nanomaterials to enhance VFAs accumulation in sludge anaerobic digestion. However, other studies also found that propionate acid was the primary fatty acid (Feng et al., 2009). This dominance might be attributed to the difference in key enzymes. The anaerobic digestion of WAS was dominated by many key enzymes. Acetic acid production was closely associated with phosphotransacetylase (PTA) and acetate kinase (AK). However, the two key enzymes of oxaloacetate transcarboxylase (OAATC) and CoA transferase were relevant to propionic acid formation (Feng et al., 2009). Moreover, the process for converting propionate into acetate could be pushed forward by microorganisms. Those observations indicated that the addition of acid-producing bacteria accelerated the activities of acid-producing enzymes and key hydrolytic activities, which led to a higher percentage of low molecular weight organic acids (acetic acid and propionic acid).

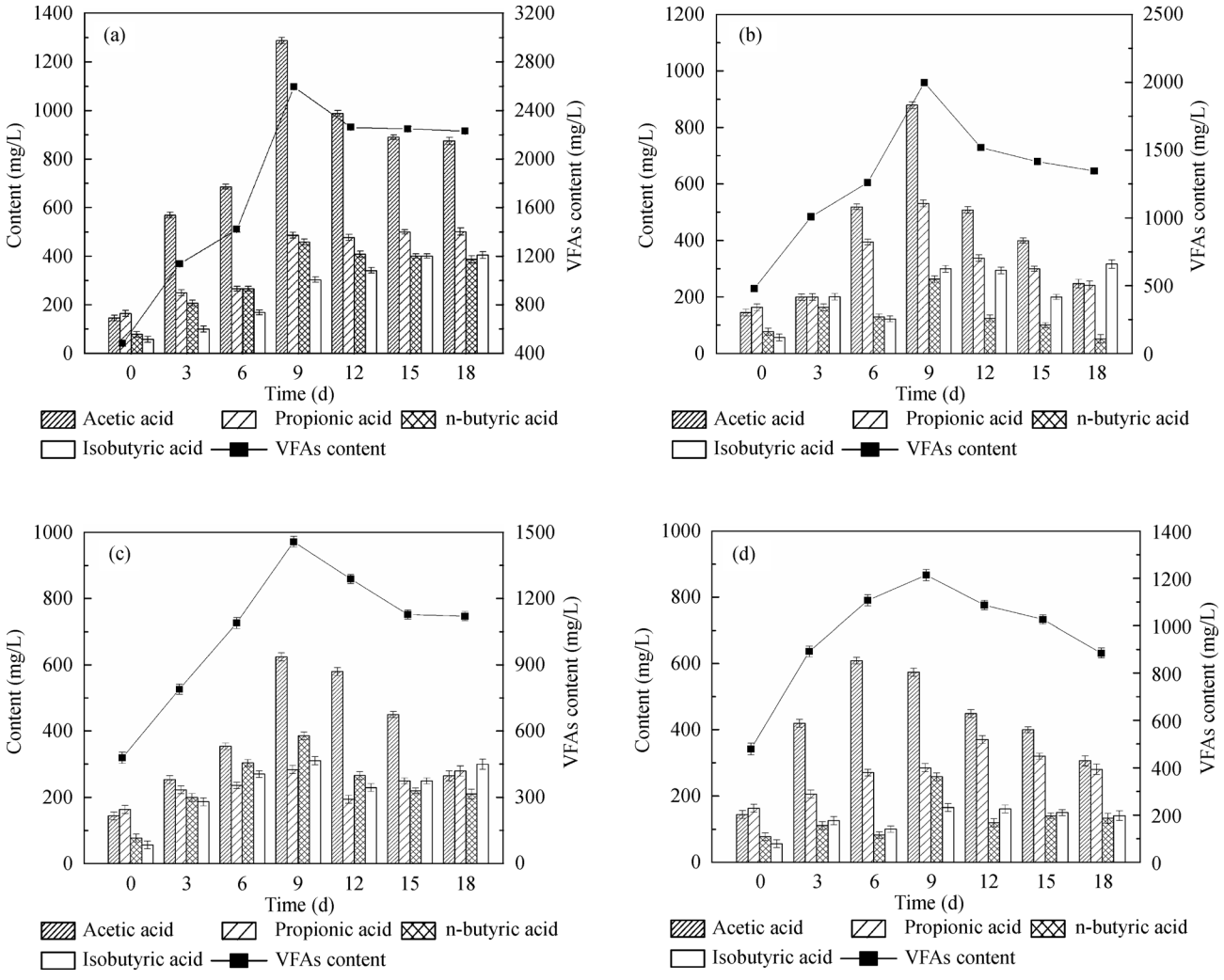

Fig. 4 Concentration of VFAs and variations in individual VFA: (a) AFB-1; (b) AFB-2; (c) AFB-3; and (d) Control group. Error bars represent standard deviations of triplicate tests

3.3.3 Effect of acid-producing bacteria on the biogas production from WAS anaerobic digestion

BMP assays represented the effect of acid-producing bacteria on the biodegradability efficiency of WAS in anaerobic digestion. Figure 5 illustrated variations in the cumulative biogas yield. During the initial 9 days of digestion, the levels of cumulative biogas production showed an upward trend whether the acid-producing bacteria were added or not, and the cumulative yield of biogas at any time had a trend of AFB-1>AFB-2>AFB-3>control group. In particular, the biogas production in the experimental groups (AFB-1, AFB-2) kept growing from the 9th day to the 12th day. However, the control group and group AFB-3 remained stable. Similar variations in biogas production were reported by Kavitha et al. (2017). This finding indicated that *Bacillus coagulans* could maintain high biogas production. The biogas production during anaerobic digestion of WAS was primarily a synergistic effect among different types of microbes. The acid-producing bacteria consumed soluble organic matter from the hydrolization stage and converted proteins and soluble carbohydrates into VFAs production. The released organics and VFAs functioned as nutrients for methanogenic bacteria, thus leading to biogas generation (Ushani et al., 2017). Therefore, these findings showed that the addition of *Bacillus coagulans* improved SCOD components and accelerated the increase in substrates for methanogenic bacteria.

As shown in Fig. 5, group AFB-1 was superior to other groups and its cumulative biogas production was 1545±40 mL. The biogas production from inoculating *Bacillus coagulans* AFB-1 increased by 90.7%. From these results, prominent biogas production was evident after inoculating *Bacillus coagulans* AFB-1. In previous studies, the biogas yield of WAS was reported by other researchers (Kuglarz et al., 2013), whose results were lower than the biogas production in this experiment. Thus, the findings showed

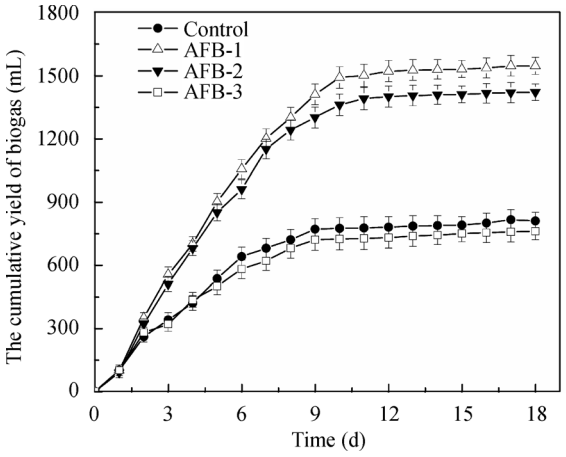

Fig. 5 Variations in cumulative biogas production. Error bars represent standard deviations of triplicate tests

that acid-producing bacteria, especially *Bacillus coagulans*, could accelerate sludge fermentation and was likely to play a more important role in anaerobic digestion of WAS.

3.4 Optimization of dosage of bacterial cells

The inoculation rate was the key to cell growth and the anaerobic digestion process. The increasing inoculation rate would enhance the degradation efficiency within a certain range. However, too much inoculation could limit the rate of cell growth due to the lack of nutrients, and it could even cause the cells to lose their ability to reproduce and lead to self-decomposition. Therefore, it resulted in low treatment efficiency. Thus, an appropriate inoculation rate was important for the degradation of WAS. In this study, the inoculation size of *Bacillus coagulans* AFB-1 ranging from 10% to 40% was performed. From Fig. 6, the influence of the inoculation rates on TCOD removal and VS removal in WAS treatment by *Bacillus coagulans* AFB-1 was examined. When the inoculation ranged from 10% to 30%, the increased inoculation enhanced the degradation efficiency. At the inoculation of 40%, the cells showed low concentration and poor activity, resulting in low treatment efficiency. Among the inoculations of 10% to 30%, the removal of VS and TCOD reached its highest at an inoculation of 30%. The biomass growth also reached its highest on the 9th day, and then the cells entered a steady decline phase. Thus, for degradation efficiency, the optimal inoculum concentration of *Bacillus coagulans* AFB-1 was 30%.

4 Conclusions

This study was focused on the potential use of newly isolated acid-producing bacteria for anaerobic digestion of

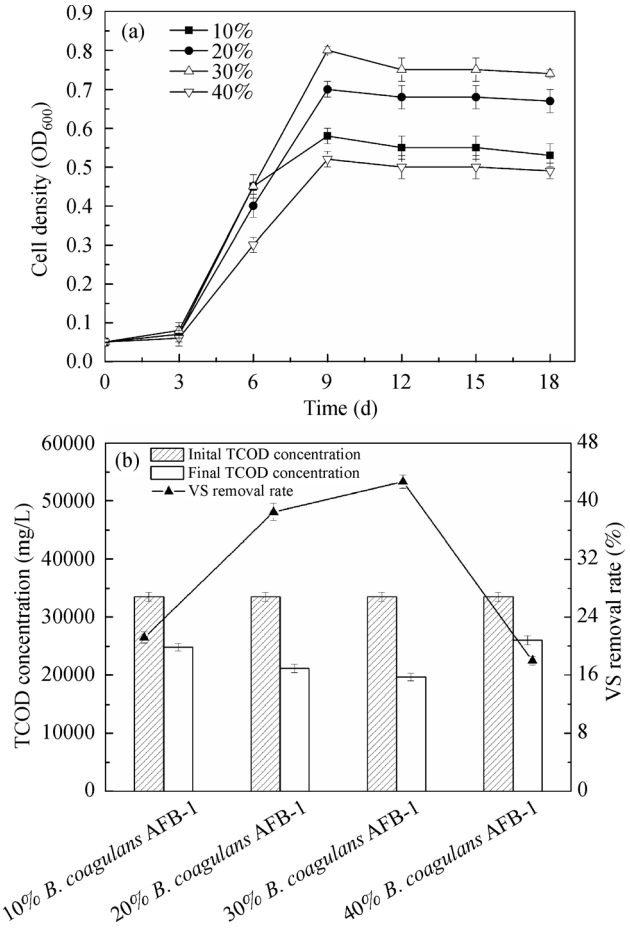

Fig. 6 Effect of inoculating *Bacillus coagulans* AFB-1 on (a) cell density, (b) TCOD removal and VS removal during anaerobically digested sludge treatment. Error bars represent standard deviations of triplicate tests

WAS. From the experimental results, it was concluded that the addition of acid-producing bacteria, especially *Bacillus coagulans* AFB-1, could promote solubilization, hydrolysis, and acidogenesis processes without apparently affecting methanogenesis. Further study showed that the optimal inoculum concentration of *Bacillus coagulans* AFB-1 was 30%. This study provides a promising approach to sludge treatment.

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