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Methane-rich biogas production from waste activated sludge with the addition of ferric chloride under thermophilic anaerobic digestion system

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Thermophilic anaerobic digestion for methane production could realize the energy recovery from waste activated sludge and pollution control simultaneously, while low methane production and unbalance between hydrolysis and methanogenesis process are often encountered. Three ferric salts, i.e. $Fe(NO_3)_3$, $Fe_2(SO_4)_3$ and $FeCl_3$ were introduced to test the potential effects on sludge anaerobic digestion performance under the thermophilic system. Enhanced methane production was achieved using $FeCl_3$ as the additive with a cumulative methane production of 117.44 $mLCH_4/gVS$, which increased by 98.9% than that in the control group (59.05 $mLCH_4/gVS$), while both $Fe(NO_3)_3$ and $Fe_2(SO_4)_3$ caused some negative effects, meaning that the availability of anion should also be considered except to iron ion. The introduction of $FeCl_3$ created a favorable environment paralleling to the positive precipitation and biocatalysis. The succession of microbial communities before and after the introduction of ferric salts was investigated and compared through pyrosequencing analysis, and especially the dominant *Methanosarcina* increased from 1.3% to 63.2% in effective reads with the addition of $FeCl_3$.

1. Introduction

Waste activated sludge (WAS) is the inevitable byproduct from wastewater treatment process, and increases greatly with the growing quantity of wastewater and more stringent sewage discharge standard.[1] Anaerobic digestion is the attractive disposal process for sludge, which could not only reduce the sludge mass and volume, but also generate a valuable energy source in the form of biogas.[2] Compared to mesophilic digestion, thermophilic anaerobic digestion has additional benefits including a high degree of waste stabilization, more thorough destruction of viral and bacterial pathogens.[3] However, the methanogenic phase is the rate-limiting step of the process due to the slow growth rate of the methanogenic bacteria, and the acceleration of hydrolysis in thermophilic anaerobic digestion resulted in a more accumulation of volatile fatty acids (VFAs), which will inhibit the methanogensis activities greatly.[4]

There are some disinhibition methods applied for the VFAs in anaerobic digestion, such as the adjusting C/N ratio,[5] employing a two-stage digestion system to separate methanogenesis stage from other stages of hydrolysis and acidification,[6] and adding trace elements.[7] Trace metals are the necessary elements for anaerobic digestion process, and it has been confirmed that the introduction of iron could bring the positive effects in the mesophilic anaerobic digestion system, which will improve the physiological and biochemical performance by preventing sulfide inhibition and controlling phosphate in wastewater and kitchen waste treatment.[8,9] During the anaerobic digestion process, sulfate and phosphate were produced from decomposition of protein, which would react with iron ion to precipitate as ferrous sulphide and phosphate iron. The formation of iron precipitates was effective to collect phosphate, which was beneficial for reducing the cost in the following processes for treating the digested effluent.[10] Besides, iron is also an essential constituent of some cofactors and enzymes which could stimulate and stabilize the biogas process performance.[11] The supplementation of ferric chemical compounds is easy to operate for anaerobic digestion, while the metal speciation and the anion might influence the operation conditions in the anaerobic reactors, such as the total metal concentration and reactor conditions (e.g. pH, oxidation-reduction potential (ORP) and temperature).[11,12] Based on the positive effects of iron, it was assumed that the introduction of ferric salts into sludge thermophilic anaerobic digestion system has a direct influence on biological oxidation, which is expected to create a more favourable environment for the methane production.

The application of trace metals in anaerobic digestion of WAS has been reported in some works, while the availability of different ferric salts and their potential routes in sludge are still limited, especially in the thermophilic anaerobic digestion system. To clarify these issues, $Fe(NO_3)_3$, $Fe_2(SO_4)_3$ and $FeCl_3$ were selected as the additives for the improvement of thermophilic anaerobic process in this study. The objectives of this work were to investigate the effects of these three ferric salts on biogas production process, anaerobic digestion conditions, and the fate of iron ion in the system. Finally, the microbial community structure and relative abundant were also

Table 1 Characteristics of the raw sludge and seed sludge.

Parameters	Raw sludge	Seed sludge
pH	6.32-6.40	6.87-6.90
TS (g/L)	39.5–39.9	68.7-69.2
VS (g/L)	29.0–29.3	49.6-52.2
TCOD (mg/L)	32,570–37,640	84,576-87,260
SCOD (mg/L)	124.0–535.6	16,240-18,440
STN (mg/L)	104.18–121.8	762.6-812.4
STP (mg/L)	55.13–98.64	416.16-467.38
C (%)	34.68–34.83	-
H (%)	5.39–5.51	-
N (%)	6.76–6.90	-
S (%)	1.35–1.38	-
Fe (%)	1.72-1.91	1.83-1.94

studied by the 454 GS-FLX pyrosequencing technology to get insight into the thermophilic anaerobic digestion system directly.

2. Experimental

2.1 Sludge sampling and characterization

WAS used in this study was obtained from the secondary sedimentation tank of a municipal wastewater treatment plant (MWWTP) in Shanghai, China, where wastewater was treated by the anaerobic-anoxic-aerobic process with a capacity of 50,000 m^3/d. The sludge obtained was screened with a 1.0-mm mesh to eliminate large particles and hair before thickening to required solid concentrations. Then the pre-treated samples were stored at 4℃ for further analyses. The seed sludge (inoculum) was collected directly from a long-term continuous lab-scale anaerobic bioreactor in our lab, fed with activated sludge. Compared to the raw sludge, the seed sludge had a healthy population of methanogens and other microorganisms needed for the efficient start-up of new digesters. The main characteristics of the raw sludge and seed sludge are shown in Table 1.

2.2 Experimental design

Batch experiments were carried out in double-walled cylindrical vessels with 6 L working volume. The seed sludge to WAS ratio was 1:3 (volume: volume). After loading the sludge, oxygen was removed from the headspace by the injection of nitrogen gas (99.99%) for 5 min to maintain anaerobic conditions. During the anaerobic digestion process, all the reactors maintained at a thermophilic digestion temperature of 55±2 ℃ by water circulation, equipped with stainless-steel stirrers for mixing the contents. The biogas was measured using a calibrated sampling syringe. All samples from the reactors were analysed in triplicate. Aim to investigate the effects of different ferric salts on the thermophilic anaerobic digestion of WAS, a fixed dosage of $Fe(NO_3)_3$, $Fe_2(SO_4)_3$ and $FeCl_3$ were applied (referred to as R2, R3 and R4, respectively) in the form of ferric salts solutions (50 mL) on 3rd day after the experimental start-up, which based on the iron ion equivalent of 200 mg/L. A control group (R1) was also carried out under the same operation conditions except to the absent of ferric salt. No alkalinity or buffering agent was added into the system, and the pH value was not adjusted during the entire process.

2.3 Analytical methods

50 mL sludge was collected from the reactors each time after completely mixed, and the corresponding supernatant was achieved by centrifugation at 12,000 rpm for 5 min with a subsequent filtration through 0.45μm microfiber filter paper. The ORP and pH were measured by an ORP meter (ORP-502, Ruosull Technology Co., Ltd., Shanghai) and a pH meter (pHs-3C, Leici Co. Ltd., Shanghai), respectively. TS, VS, soluble chemical oxygen demand (SCOD), NH_4^+-N, total nitrogen (TN) and soluble phosphorus (TP) were measured according to Standard Methods.[13] VFAs (including acetic acid, propionic acid, butyric acid, iso-butyric acid, valeric acid and iso-valeric acid) were analyzed by a gas chromatograph (GC-2010, Shimadzu) with a chromatographic column (DB-FFAP：30 m ×0.25 mm×0.25 mm) and a flame ionization detector (FID). The concentrations of CH_4 and CO_2 were quantified by a gas chromatograph (GC-14B, Shimadzu) equipped with a chromatographic column (TDX-02) and a thermal conductivity detector (TCD).[14] The concentration of iron ion in the supernatant was conducted with atomic absorption spectrometry method (spectrometer contrAA, Analytik Jena). Energy-dispersive spectrometer (EDS) analysis of the sludge samples was performed by a large area of silicon drift spectrometer detector (Large Area SDD, AZtec X-Max 80). The contents of C, H, N and S in WAS were measured by an elemental analyzer (Vario Macro Cube, Elementar) with sulfanilamide ($C_6H_8O_2N_2S$) as reference material. All the results were a meaning value in triplicate with an accuracy of ±5%.

2.4 DNA extraction and high-throughput 16S rDNA gene pyrosequencing

Mixed sludge was collected from the reactors before start-up and 28 days operation when daily methane production (DMP) was in a high level. The samples were washed with phosphate-buffered saline, after which the genomic DNA of the samples was extracted using an extraction kit (Felix bio-tech, USA) according to the manufacturer's instructions. The quality of the extracted DNA was checked by determining its absorbance at 260 and 280 nm, and Agarose gel electrophoresis (AGE) was employed to test the DNA integrity.

Two universal primers for archaeal, 787F (5'-ATTAGATACCCSBGTAGTCC-3') and 1059R (5'-GCCATGCACCWCCTCT-3') were used to amplify archaeal 16S rRNA gene.[15] The PCR program consisted of an initial 5 min denaturation step at 94℃, 27 cycles of repeated denaturation at 94℃ for 30 s, annealing at 54℃ for 30 s, and extension at 72℃ for 30 s, followed by final extension step of 5 min at 72℃. After being purified and quantified, the PCR products of 16S rRNA gene was determined by pyrosequencing using the Roche 454 FLX Titanium sequencer (Roche 454 Life Sciences, Branford, CT, USA) according to the methodology described by Zhang et al.[16] Subsequently, the MOTHUR program was used to cluster effective sequences into operation taxonomic unit (OTU) by a 3% level. Rarefaction curves, species richness estimator of Chao1 and Shannon diversity index were conducted by MOTHUR to identify the species diversity for each sample. The OTUs defined by a 3% distance level were classified using the RDP classifier at a 50% confidence threshold. The effective sequences obtained from pyrosequencing were compared with Greengenes 16S rRNA gene database using NCBI's BLASTN tool, and the species distribution diagram was employed. In order to further distinguish the dominant species who should account for the

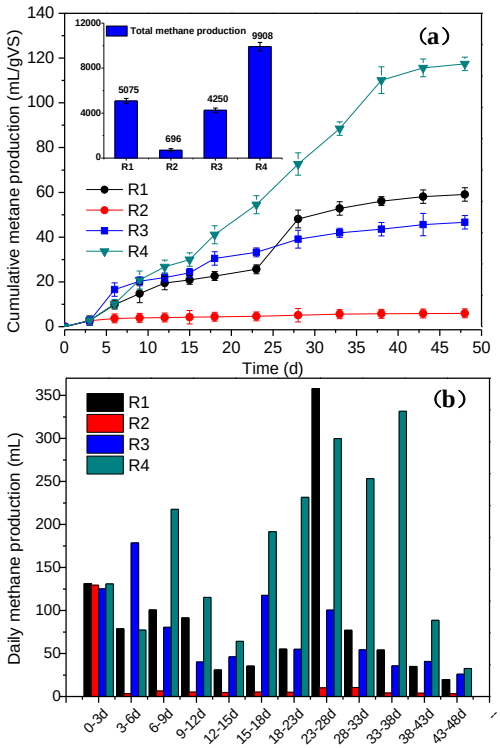

Fig. 1 (a) Cumulative methane production (CMP) and (b) Daily methane production (DMP) during the anaerobic digestion process.

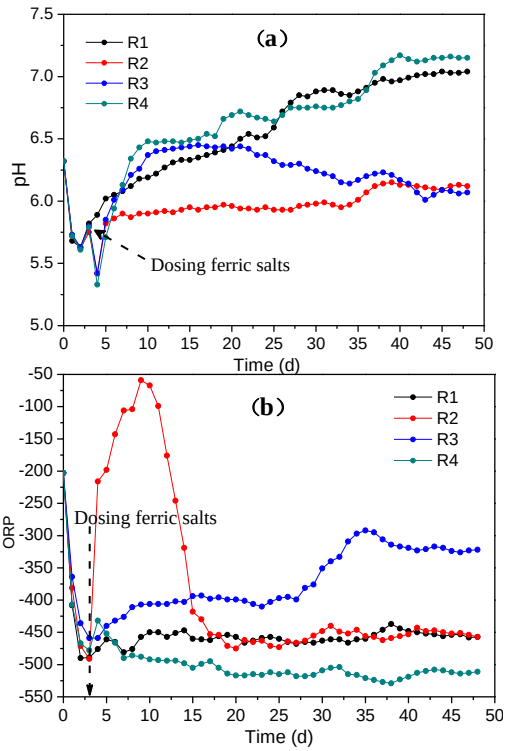

Fig. 2 (a) Variations of pH during the anaerobic digestion process; (b) Variations of ORP during the anaerobic digestion process.

different digestion performances among the reactors, phylogenetic relationships of sequences were conducted according to the method described by Ye et al. [17] The main software MEGAN was used for metagenome analysis, which could conduct cluster analysis for the extensive sequencing data.

3. Results and discussion

3.1 Biogas generation performance

To investigate the effects of ferric salts on the thermophilic anaerobic digestion of WAS, DMP, total methane production (TMP) and cumulative methane production (CMP) which based on the initially loaded volatile solid (VS) were determined and calculated.

Fig 1a illustrates the time curves of the CMP of the reactors (R2, R3 and R4) with dosing $Fe(NO_3)_3$, $Fe_2(SO_4)_3$ and $FeCl_3$ and the control group (R1), respectively. In the initial stage, R1, R3 and R4 had a similar tendency, while that in R2 with dosing $Fe(NO_3)_3$ had no distinct increase. Afterwards, CMP increased greatly in R4 with dosing $FeCl_3$ and reached the highest peak around 43rd day; and thus a final CMP of 117.44 $mLCH_4/gVS$ was obtained, which was 98.9% higher than that in R1 (59.05 $mLCH_4/gVS$) and similar to the values (102-145 $mLCH_4/gVS$) reported by Sheets et al under the thermophilic anaerobic system.[4] It should be emphasized that it is still lower than that in some researches and even the maximum CMP of 256.45 $mLCH_4/gVS$ was achieved after the thermal pre-treatment (70 ℃),[18] which might be due to the differences of sludge and inoculum properties, operating conditions (reactors, hydraulic retention time (HRT), pH). For R2 with the addition of $Fe(NO_3)_3$, the biogas production was inhibited completely, and a low CMP of 5.96 $mLCH_4/gVS$ was observed. With dosing $Fe_2(SO_4)_3$, a stable increase of CMP was obtained during the process, and finally reached a peak of 46.67 $mLCH_4/gVS$. The maximal TMP was also found in R4, 95.23% higher than that of R1 (5075 mL); whereas the TMP of R2 and R3 were only 695 mL and 4250 mL, respectively. Nevertheless, the addition of $FeCl_3$ could contribute to enhance the methane production from WAS under the test conditions, which confirmed the positive effects of iron on anaerobic digestion.[19]

With respect to DMP (Fig. 1b), all reactors had a rapid methane production rate in the first three days, meaning that methanogenesis was effectively activated in the reactors,[20] and then DMP in R1, R3 and R4 declined around 9th day due to the excessive accumulation of VFAs and low pH resulting from rapid hydrolysis acidification in the thermophilic anaerobic digestion;[21] DMP increased and reached peaks afterwards. DMP of R2 was low in the whole process as a result of the unsuitable conditions for methanogens. As an indicator for process stability, the ratio of CO_2/CH_4 (Fig. A1†) in R2 kept in a high level (>3), indicating the small proportion of CH_4 in biogas, which confirmed the process failure after dosing $Fe(NO_3)_3$.[14]

3.2 Variations of anaerobic digestion conditions

It is well known that sludge anaerobic digestion is a delicate balance between the rate of hydrolysis and methanogenesis because of the far more sensitivity of methanogenic bacteria than acidogenic and fermentative bacteria to VFAs accumulation and pH drop.[22] Hence, the key factors: pH, ORP and concentrations of VFAs, SCOD, TN and NH_4^+-N were measured to assess the process stability of anaerobic digestion.[23]

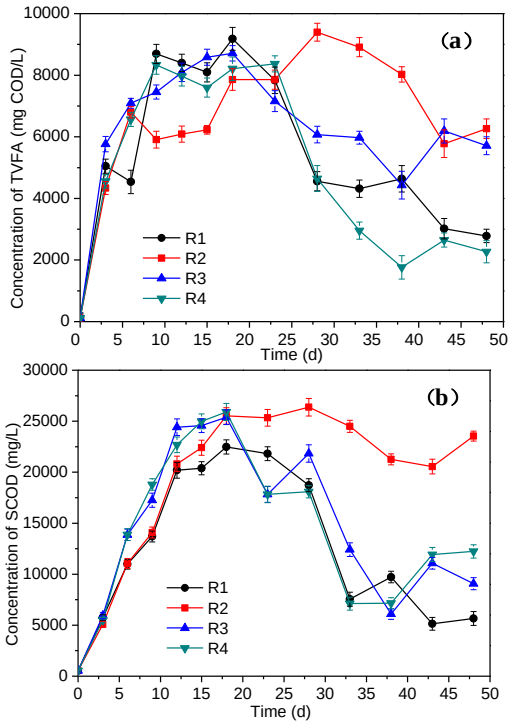

Fig. 3 (a) Variations of VFAs concentrations in the supernatant; (b) Variations of SCOD concentrations in the supernatant.

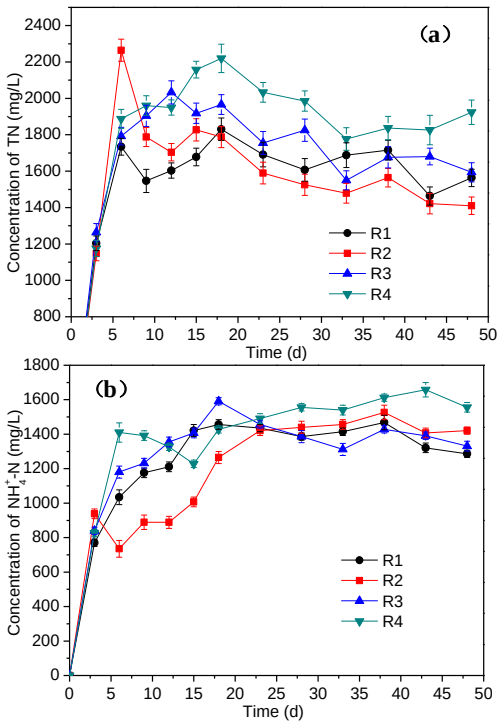

Fig. 4 (a) Variations of TN concentrations in the supernatant; (b) Variations of NH_4^+-N concentrations in the supernatant.

3.2.1 Variations of pH and ORP

Variations of pH and ORP in the reactors were showed in Fig. 2. After the commencement of the experiment, pH fell immediately and reached a minimal value of 5.8 within the first 3 days due to hydrolysis acidification.[20] When different ferric salts were dosed into R2, R3 and R4, pH sharply dropped to 5.3-5.4, combined with the acidification property of these three ferric compounds and the bond of the hydroxyl ion through the settlement process. After the rapid decline, pH values in R1 and R4 increased and remained in the range of 6.5-7.2, which nearly lied in the desired pH range of 6.8-7.2 for anaerobic digestion.[24] The pH value of R2 kept an inconspicuous fluctuation below 6.2, which was corresponding with the poor methane production. Similarly, the biggest change of ORP was observed in R2, which increased sharply from -491 mV to -59 mV after dosing $Fe(NO_3)_3$ due to the strong oxidizing property of ferric and nitrate ions; and the failure of methanogenesis in R2 might attribute to the huge shock and the sensitivity of methanogens to the environmental condition. The ORP levels in R1 and R4 continued to decline and fluctuated in a small range, whereas that in R4 was relatively lower (from -505 mV to -527 mV) which was more favorable for the thermophilic methanogenic bacteria.[25] Particularly, ORP in R3 increased rapidly from -403 mV to -295 mV (from day 24 to 36) and then slowly dropped to -322 mV in the end, which was corresponding well with the gradual methanogenesis inhibition. As the cell rupture and proteolysis, SO_4^{2-} was constantly released into the supernatant. Since the introduction of SO_4^{2-} in R3, iron ion was not enough for the sulfide control and resulted in the enrichment of sulfate-reducing bacteria (SRB). SRB will compete with methanogens for the common substrates hydrogen, formate and acetate, and in general they have better growth kinetic properties than methanogens in the presence of sulfate.[26] The oxidation of organics by SRB might account for the higher ORP in R3, which was adverse to the growth of methanogens and accounted for the worse biogas production afterwards.[8,27]

3.2.2 Effect of different ferric salts on VFAs and SCOD

Variations of total VFA (TVFA) in the supernatant (Fig. 3a) were in accordance with the pH, and the hydrolysis of sludge particles and the rapid growth of acidogenic and fermentative bacteria seemed to be implemented in a rapid way, which led to the VFAs accumulation immediately. On the whole, the concentrations of TVFA in all the reactors firstly experienced a sharp increase during the initial digestion period, then a moderate variation from day 9 to 23, followed by steady decline except to that in R4 fluctuating in a high level (from 6260 to 9396 mg/L). The maximal TVFA concentration was obtained on 18th, 28th, 15th and 23rd in R1, R2, R3 and R4, which were 9183, 9396, 8582 and 8370 mg/L, respectively. Meanwhile, the TVFA concentration in R4 reduced relatively stable, meaning that a balance was established between acidification process and methanogenic activity. For the constituents of TVFA (Fig. A2†), the dominant constituent of acetic acid could be referred as the indicator for the TVFA concentration, and it decreased to zero in R4 at the end of the experiment.[7] The effective conversion of VFAs in R4 resulted in the favorable pH and enhanced biogas production.

SCOD of R1, R3 and R4 (Fig. 3b) increased sharply first then kept a small fluctuation, followed by a steady decline, as same as the trend of TVFA curve;[28] whereas that in R2 kept at a high level ranged from 20550 to 26374 mg/L due to the poor methanogenesis. After the rapid growth of SCOD, the peak values were obtained on 18th in each reactor, which were 22483, 25522, 25391 and 25912 mg/L, respectively. Obviously, the

Table 2 Biodiversity estimation of 16S rRNA gene libraries from the pyrosequencing analysis.

Sample	Effective reads	Observed OTUs	Estimated OTUs by chao1	Shannon
R0	12,340	412	886	3.375
R1	17,985	323	689	2.531
R2	14,992	367	1,076	3.134
R3	18,372	305	536	2.756
R4	18,998	230	570	1.767

growth rate of R4 was the fastest in all reactors, and a rapid decline was obtained with the enhanced methane production rate, indicating that the addition of $FeCl_3$ could accelerate the conversion of soluble organic matters in the form of biogas. On the other hand, the control group (R1) maintained a lower SCOD than the other three reactors in the later digestion period, and the concentration ranged from 5137 to 5665 mg/L.

3.2.3 Effect of different ferric salts on TN and NH_4^+-N in the supernatant

In thermophilic anaerobic digestion system, the decomposition of protein in heat-labile microbes and extracellular polymeric substances gave rise to concentration of TN in initial stage as shown in Fig. 4(a). The concentration of TN in R3 and R4 had a rapid increase after dosing $Fe(NO_3)_3$ and $FeCl_3$, and a relatively high level was achieved in R4 during the whole process, which was in accordance with the variations of SCOD and VFAs and confirmed the acceleration effect of ferric salts on hydrolysis and acidification process. A rapid increase of TN was observed in R2 due to the introduction of nitrate (~664 mg/L) with the highest value of 2265 mg/L, followed by a declined trend. As shown in Fig. 4(b), the similar trend of variations of NH_4^+-N in R3 and R4 was observed, while that in R2 dropped rapidly to 735 mg/L, and then ascended steadily. It could be explained by the existence of anaerobic ammonium oxidation (ANAMMOX) as reaction (1):

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2 \quad (1)$$

Theoretically, 1.32 moles NO_2^- will consume 1 mole NH_4^+ with 1.02 mole N_2 and 0.26 mole NO_3^- productions.[29] Due to the introduction of NO_3^-, the NO_3^- conversion from NO_2^- was inhibited during the nitrification process, which resulted in the accumulation of NO_2^- and the decrease of NH_4^+ by ANAMMOX reaction.

3.3 Pyrosequencing analysis of microbial community

3.3.1 Biodiversity of microbial community phylotypes

Mixed sludge sample before start-up (referred to as R0) and sludge samples taken from R1, R2, R3 and R4 on 28th were collected and used for investigating the succession of microbial communities before and after the introduction of ferric salts by pyrosequencing analysis. As shown in Table 2, a total amount of 12,340 (R0), 17,985 (R1), 14,992 (R2), 18,372 (R3) and 18,998 (R4) effective sequence tags were obtained through primer and barcodes matching with raw reads and a series of filtering process. The observed number of operational taxonomic units (OTUs) at a 3% distance were 412 (R0), 323 (R1), 367 (R2) 305 (R3) and 230 (R4), respectively. Chao1, as a metric for species richness were investigated, which integrated with the corresponding Shannon index for presenting the species diversity, jointly implied that some microbial communities were enriched selectively.[30]

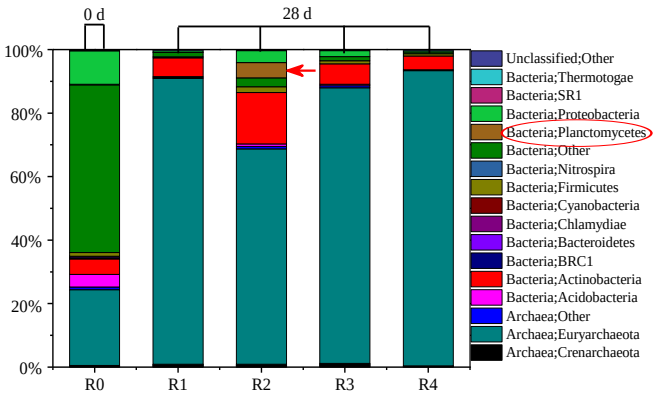

Fig. 5 Taxonomic classification of the dominant phylogenetic groups at the phylum level (The relative abundance of phyla less than 1% of total composition in the five libraries was defined as "other").

3.3.2 Bacteria and archaea taxonomy analyses

To clarify the difference of species diversity, the relative microbial community abundances were identified on the phylum level (Fig. 5). *Euryarchaeota*, *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, *Firmicutes*, *Crenarchaeota*, *Bacteroidetes*, *BRC1* and Unclassified bacteria were dominated in these five samples, and the main difference was attributed to the distribution of *Euryarchaeota*, *Acidobacteria*, *Actinobacteria*, *Proteobacteria* and unclassified bacteria. As digestion continued, the microbial community structure achieved a significant shift in R1, R2, R3 and R4. The relative abundance of unclassified bacteria in R0 was 52.8%, which was significantly higher than that in other samples (0.70%, 3.80%, 1.90% and 0.60%, respectively). *Actinobacteria*, as the main fermentative bacteria, had a significant increase in R4 (16.2%) compared to R0 (4.9%); whereas that in R1, R2, R3 was 5.9%, 6.4% and 4.4%, respectively, contributing to the rapid increase of SCOD and VFAs in R4.[31] Additionally, the relative abundance of *Planctomycetes* in R2 (4.8%) was the highest, while that in other samples were very low (near to zero). As reported, *Planctomycetes* as the main chemoautotrophic bacteria for ANAMMOX process could oxidize ammonium with nitrite as the electron acceptor and with CO_2 as the main carbon source. This result identified the existence of ANAMMOX after dosing $Fe(NO_3)_3$ in the thermophilic anaerobic digestion system, and explained the variation of NH_4^+-N in the early stage.[32] As for archaea, *Methanogens* was in the dominant status, and the highest relative abundance was obtained in R4 (93.3%), followed by R1 (91.3%), R2 (74.2%) and R3 (88.4%), respectively.

The sequences from archaea were also analysed at the genus level as shown in Table 3, and a total amount of 10 genera was identified as the main composition in the five samples. *Methanosarcina* presented a highest relative abundance in R4 (63.2%) as compared with that in R0 (1.3%), R1 (35.7%), R2 (2.7%) and R3 (31.4%), which were corresponding well with the methane production. The relative abundance of genera *Methanosphaerula* and *Methanomethylovorans* had no apparent distinction among R1, R2, R3 and R4, and compared with R4

Table 3 Taxonomic classification of archaea based on the effective reads at 3% distance at the genus level.

Genus	R0	R1	R2	R3	R4
Methanobacterium	1.60%	4.30%	3.20%	3.30%	1.20%
Methanobrevibacter	6.00%	2.60%	1.60%	1.90%	0.50%
Methanothermobacter	2.60%	12.60%	1.80%	6.20%	3.00%
Methanoculleus	0.20%	1.00%	4.30%	1.30%	5.00%
Methanosphaerula	1.90%	0.70%	0.70%	0.60%	0.60%
Methanolinea	0.10%	0.80%	1.40%	1.00%	0.20%
Methanospirillum	1.40%	6.50%	10.40%	7.70%	2.10%
Methanosaeta	4.10%	17.40%	16.20%	16.70%	5.00%
Methanomethylovorans	0.90%	0.30%	0.20%	0.20%	0.20%
Methanosarcina	1.30%	35.30%	2.70%	31.40%	63.20%
Others	5.30%	9.80%	31.70%	18.10%	12.30%
Archaea	**25.40%**	**91.30%**	**74.20%**	**88.40%**	**93.30%**

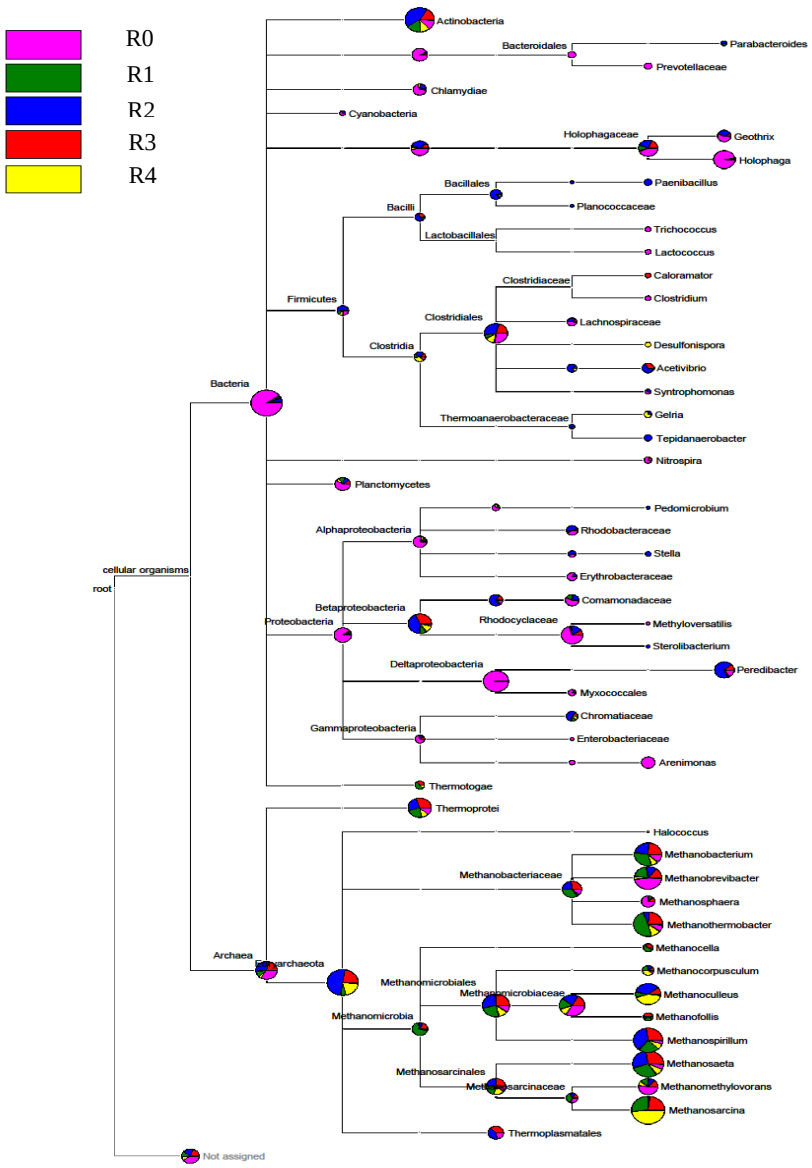

Fig. 6 Phylogenetic relationships of sequences from the dominant bacterial communities in the five samples.

Table 4 The main functions of ferric salts used in the wastewater treatment.

Ferric salts Species	Treatment Objective	Dosage (mg/L)	Wastewater Categories	Functions	Removal Efficiencies	Ref.
$FeCl_3$, $Fe_2(SO_4)_3$	Biomass removal	150-250	Piggery wastewater	Coagulation, Flocculation	66-98%	34
$FeCl_3$	Sulfide control	0.4-5.4	Domestic wastewater	Oxidation, Precipitation	—	35
$FeCl_3$	Phosphorus control	0.44	Domestic sewage	Precipitation	100%	10
$FeCl_3$	COD removal	100-200	Slaughterhouse wastewater	Precipitation	45-75%	36
$FeCl_3$	Color removal	3500	Molasses wastewater	Co-precipitation	96%	37

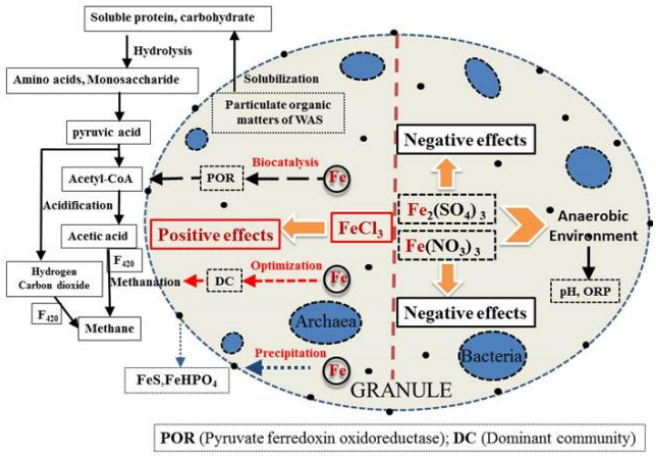

Fig. 7 Possible mechanisms of different ferric salts in the thermophilic anaerobic digestion of WAS (Only the main methanogenesis process and key enzyme assayed are labelled).

the higher relative abundance of genera *Methanobacterium*, *Methanobrevibacter*, *Methanolinea*, *Methanospirillum* and *Methanosaeta* in R1, R2 and R3 were identified. Meantime, the genus *Methanoculleus* was enriched in R4 with the highest relative abundance (5%) as compared with that of 0.2%, 1%, 4.3% and 1.3% in R0, R1, R2 and R3, respectively.

Phylogenetic relationships of sequences from the dominant microbial community (bacteria and archaea) of each sample were shown in Fig 6. These sequences were assigned into NCBI taxonomies with BLAST and MEGAN.[16] Pie charts indicated the relative abundance of each phyla, class, family and genus. The relative abundances of the corresponding phyla, class, family and genus in the five samples were defined as the ratio of each colour area to pie area. Although most of the genera were found simultaneously in each sample, there were still differences in terms of the relative abundance. The main bacteria in R0 was genus *Proteobacteria*, while that in R1, R2, R3 and R4 was genus *Firmicutes*, indicating that the genus *Firmicutes* was enriched and the dominant status of genus *Proteobacteria* was weakened during the digestion process. Some specific species of archaea were selectively enriched,[33] and genera *Methanobacterium*, *Methanothermobacter* and *Methanosaeta* were the dominant communities in R0 and R1, while that in R2 were genera *Methanoculleus*, *Methanospirillum* and *Methanosaeta*. It should be emphasized that genera *Methanosarcina* and *Methanoculleus* were absolute dominant communities in R4 (marked by yellow color), which accounted for the higher methane production.

3.4 Possible mechanisms analysis

Ferric salts were widely used in wastewater treatment for the outstanding performances of sulfide and phosphorus control, COD and color removal as shown in Table 4. Similarly, it was found that the addition of ferric salts could affect the thermophilic anaerobic digestion of WAS significantly, and $FeCl_3$ was found to be the best one for the enhancement of methane production with the increase rate of 98.9% under the test conditions. The introduction of $FeCl_3$ in R4 might be helpful for the establishment of a balance between the hydrolysis process and methanogenous phases. As shown in Fig 3b, rapid hydrolysis and acidification took place in the early stage, and SCOD was converted to VFAs; most of the VFA was acetic acid (Fig. A2†), which was the suitable substrate for methanogenesis,[7] and then VFAs generated were immediately converted to methane. In general, Fe(III) is reduced to Fe(II) chemically or biologically by iron-reducing bacteria (IRB) during the anaerobic digestion process according to Iron Pourbaix diagram,[38] hence the iron in the anaerobic system was mainly in the form of ferrous ion.[39] The concentration of iron ion in the supernatant was conducted and there were no apparent distinction among the reactors (data not shown), which indicated the precipitation formation of iron (e.g. ferrous sulfide and monobasic ferrous phosphate) and adsorbed in the granule, as reported by Ge et al. and Firer et al.[9,40] Also, the sludge matrix absorption of metals at specific sites was intensive due to the high porosity and large internal surface area of granules,[41] and it was confirmed according to EDS profile in Fig. A3†. The formation of FeS reduced the release of H_2S, which was toxic to methanogens, and it might be one reason for the methane performance.[42] The contents (%) of sulfur in dry sludge solid before and after the anaerobic digestion were investigated, and the higher sulfur content in R2 (1.187%), R3 (1.399%) and R4 (1.277%) was obtained, compared with that in the control group (1.128%), confirmed the effect of iron ion for sulfur control.

In addition, iron was an indispensable component of pyruvate-ferredoxin oxidoreductase (POR) which can catalyze pyruvate catabolism with the production of CO_2 and acetyl-CoA, meaning that iron could have a direct influence on biological oxidation, and thus the methane production was improved. In this study, it was found that the addition of $FeCl_3$ into thermophilic anaerobic digestion system could optimize microbial community structure and the possible mechanisms of iron were shown in Fig. 7. After dosing $FeCl_3$ into R4, special bacteria and archaea were selectively enriched, namely, the dominant communities shifted from *Bacteroidetes* and *Proteobacteria* to *Firmicutes*, and especially the main archaea identified in R4 was *Methanosarcina*. According to the reports,

Methanosarcina is the only one which could utilize all the three methane production pathways (aceticlastic methanogenesis pathway, hydrogenotrophic methanogenesis pathway and methyl compound utilizing pathway) and tolerate different adversity factors, such as temperature variation or high concentration of acetic acid.[30] When the dominant status of methanogens formed, VFAs as the main products of hydrolysis acidification were degraded to methane timely; then the pH increased and a favorable environment was created for the stable methanation.

It should be emphasized that both of the anions and cations in ferric salts should be considered, which has been observed in this work that not all the ferric salts could contribute to the methane production because the addition of ferric salts is not only the introduction of micronutrients for microorganisms, but also creates a special environment for anaerobic digestion. Specially, the introduction of NO_3^- resulted in the low pH and high ORP, accompanied with the AMMNOX, which was adverse to the methanogens. In terms of SO_4^{2-}, the introduction could be beneficial to the oxidation of organics by SRB, but the simultaneous competition for common organic and inorganic substrates also resulted in the gradual methanogenesis inhibition. Nevertheless, the addition of $FeCl_3$ could enhance the methane production in the thermophilic anaerobic digestion system, which should attribute to the positive effects of iron and favorable anaerobic conditions created.

4. Conclusions

Among the three test ferric salts, $FeCl_3$ additive was the best one for enhancing the methane production from WAS, which was 98.9% higher than the control group. The direct reason for the enhanced methane production was the favorable environment through assessing the key factors of pH, ORP and variations of VFAs, SCOD, TN and NH_4^+-N. Interestingly, the introduction of NO_3^- resulted in the low pH and high ORP, accompanied with the AMMNOX, and also the gradual methanogenesis inhibition occurred with the addition of $Fe_2(SO_4)_3$. In the thermophilic anaerobic system, apart from the positive effects of precipitation of sulfide and biocatalysis for methanogenesis process, the optimization on microbial community structure was also identified. Pyrosequencing analysis of microbial communities before and after the introduction of ferric salts showed that some specific species of bacteria and archaea were selectively enriched, and the enhanced methane production in $FeCl_3$ additive group should attribute to the enriched *Actinobacteria* for hydrolysis-acidification and *Methanosarcina* for methanogenesis with the values of 16.2% and 63.2%, respectively.

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Notes and references

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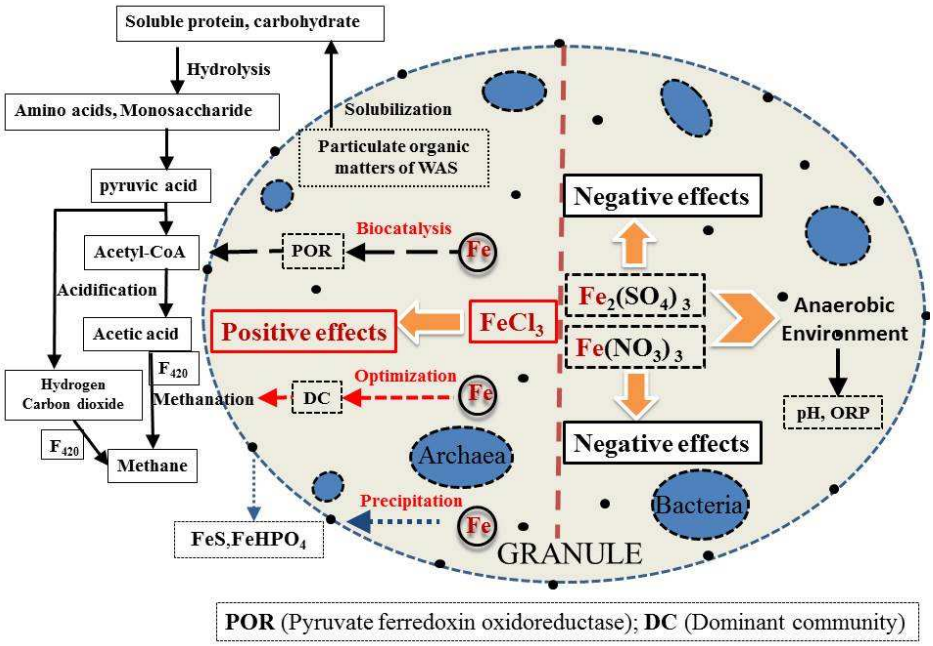
† Electronic Supplementary Information (ESI) available: The ratio of CO_2/CH_4, Variations of VFAs (including acetic acid, propionic acid, butyric acid, iso-butyric acid, valeric acid and iso-valeric acid), EDS analysis of the sludge samples. See DOI: 10.1039/b000000x/

1 R. R. Wang, M. J. Eckelman and J. B. Zimmerman, *Environ. Sci. Technol.*, 2013, **47**, 11189-11198.
2 O. Thygesen, S. G. Sommer, S. G. Shin and J. M. Triolo, *Fuel*, 2014, **132**, 44-46.
3 K. V. Lo, P. H. Liao and A. C. March, *Biomass*, 1985, **6**, 301-315.
4 J. P. Sheets, X. M. Ge and Y. B. Li, *Bioresour. Technol.*, 2015, **180**, 296-303.
5 F. X. Ye, Y. Y. Ye and Y. Li, *J. Hazard. Mater*, 2011, **188**, 37-43.
6 Y. Xia, H. H. Fang and T. Zhang, *RSC Adv.*, 2013, **3**, 15528-15542.
7 G. Y. Zhen, X. Q. Lu, J. Niu, L. H. Su, X. L. Chai, Y. C. Zhao, Y. Y. Lin, Y. Song and D. J. Niu, *Chem. Eng. J.*, 2013, **233**, 274-281.
8 L. Zhang and D. Jahng, *Waste Manage*, 2012, **32**, 1509-1515.
9 H. Q. Ge, L. S. Zhang, D. J. Batstone, J. Keller and Z. G. Yuan, *J. Environ. Eng.*, 2013, **139**, 594-601.
10 O. Gutierrez, D. Park, K. R. Sharma and Z. G. Yuan, *Water Res.*, 2010, **44**, 3467-3475.
11 H. P. V. Leeuwen, *Environ. Sci. Technol.*, 1999, **33**, 3743-3748.
12 J. Gustavsson, S. S. Yekta, C. Sundberg, A. Karlsson, J. Ejlertsson, U. Skyllberg and B. H. Svensson, *Appl. Energ.*, 2013, **112**, 473-477.
13 APHA, *Standard methods for the examination of water and wastewater*, 20st ed, 1998.
14 B. Yu, J. B. Xu, H. P. Yuan, Z. Y. Lou, J. X. Lin and N. W. Zhu, *Fuel*, 2014, **130**, 279-285.
15 Y. Yu, C. Lee, J. Kim and S. Hwang, *Biotechnol. Bioeng.*, 2005, **89**, 670-679.
16 J. X. Zhang, Y. B. Zhang, X. Quan and S. Chen, *Water Res.*, 2013, **47**, 5719-5728.
17 L. Ye, M. F. Shao, T. Zhang, A. H. Y. Tong and S. Lok, *Water Res.*, 2011, **45**, 4390-4398.
18 H. N. Gavala, U. Yenal, I. V. Skiadas, P. Westermann and B. K. Ahring, *Water Res.*, 2003, **37**, 4561-4572.
19 D. Mamais, P. A. Pitt, Y. W. Cheng, J. Loiacono and D. Jenkins, *Water Environ. Res.*, 1994, **66**, 912-918.
20 H. Bouallagui, Y. Touhami, R. B. Cheikh and M. Hamdi, *Process Biochem.*, 2005, **40**, 989-995.
21 X. G. Liu, B. Dong and X. H. Dai, *Bioresour. Technol.*, 2013, **148**, 461-466.
22 A. Veeken and B. Hamelers, *Bioresour. Technol.*, 1999, **69**, 249-254.
23 S. Xie, P. G. Lawlor, J. P. Frost, Z. Hu and X. Zhan, *Bioresour. Technol.*, 2011, **102**, 5728-5733.
24 Y. Santosh, T. R. Sreekrishnan, S. Kohli, V. Rana, *Bioresour. Technol.*, 2004, **95**, 1-10.
25 T. Jash and D. N. Ghosh, *Energy*, 1996, **21**, 725-730.
26 J. Weijma, A. J. M. Stams, L. W. H. Pol and G. Lettinga, *Biotechnol Bioeng.*, 1994, **28**, 355-367.
27 H. Harada, S. Uemura and K. Monomoi, *Water Res.*, 1994, **28**, 355-367.

28 Y. S. Wong, T. T. Teng, S. A. Ong, N. Morad and M. Rafatullah, *RSC Adv.*, 2014, **4**, 64659-64667.
29 N. Chamchoi, S. Nitisoravut and J. E. Schmidt, *Bioresour. Technol.*, 2008, **99**, 3331-3336.
30 T. M. Gihring, S. J. Green and C. W. Schadt, *Environ. Microbiol.*, 2011, **14**, 285-290.
31 J. D. Vrieze, T. Hennebel, N. Boon and W. Verstraete, *Bioresour. Technol.*, 2012, **112**, 1-9.
32 Y. Kimura, K. Isaka and F. Kazama, *Bioresour. Technol.*, 2011, **102**, 4390-4394.
33 D. Y. Cheong and C. L. Hansen, *Appl. Microbiol. Biot.*, 2006, **72**, 635-643.
34 I. D. Godos, H. O. Guzman, R. Soto, P. A. Garc á-Encina, E. Becares, R. Mu ñoz and V. A. Vargas, *Bioresour. Technol.*, 2011, **102**, 923-927.
35 A. H. Nielsen, P. Lens, J. Vollertsen and T. H. Jacobsen, *Water Res.*, 2005, **39**, 2747-2755.
36 L. A. N úñez, E. Fuente, B. Mart ínez and P.A. Garc á, *J. Environ. Sci. Health.*, 1999, **34,** 721-736.
37 Z. Liang, Y. X. Wang, Y. Zhou and H. Liu, *Chem. Eng. J.*, 2009, **152**, 88-94.
38 H. A. M. Casillas, D. Cocke, J. A. G. Gomes, P. Morkovsky, J. R. Parga and E. Pterson, *Sep. Purif. Technol.*, 2007, **56**, 204-211.
39 S. C. Peng, J. Xue, C. B. Shi, J. Wang, T. H. Chen and Z. B. Yue, *Fuel*, 2014, **117**, 1-4.
40 D. Firer, E. Friedler and O. Lahav, *Sci. Total Environ.*, 2008, **392**, 145-156.
41 Y. Liu, H. L. Xu, S. F. Yang and J. H. Tay, *Water Res.*, 2003, **37**, 661-673.
42 Y. Chen, J. J. Cheng and K. S. Creamer, *Bioresour. Technol.*, 2008, **99**, 4044-4064.

Graphical Abstract



Three ferric salts, i.e. $Fe(NO_3)_3$, $Fe_2(SO_4)_3$ and $FeCl_3$ were introduced to test the potential effects on sludge anaerobic digestion performance under the thermophilic system. It revealed that the supplementation of $FeCl_3$ could create a favorable environment for methanogenesis process with enriched *Actinobacteria* for hydrolysis-acidification and *Methanosarcina* for methanogenesis, while some negative effects were observed after the supplementation of $Fe(NO_3)_3$ and $Fe_2(SO_4)_3$.