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Effect of the dosage of ferroferric oxide on batch anaerobic treatment of high strength synthetic wastewater

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ABSTRACT

Direct interspecies electron transfer (DIET) plays an important role in anaerobic wastewater treatment processes, and the dosage of conductive materials can enhance DIET. In this study, tryptone and starch were used to acclimate anaerobic sludge with different microbial communities. Then, the effect of ferroferric oxide (Fe_3O_4) dosage on batch anaerobic treatment of synthetic wastewater was examined. During methanogenesis for the tryptone acclimated anaerobic sludge, the lag phase was shortened, and the maximum methane (CH_4) production rate was increased with the dosage of Fe_3O_4. While for the starch acclimated anaerobic sludge, the CH_4 production was less affected by the dosage of Fe_3O_4. Furthermore, the dosage of Fe_3O_4 had limited effects on both hydrolysis/acidification of tryptone and methanogenesis of acetate for the tryptone acclimated anaerobic sludge. *Methanosarcina* (66.28% of archaea) and *Methanosaeta* (19.56% of archaea) were detected methanogens in the tryptone acclimated anaerobic sludge, which could accept electrons via DIET. While *Methanobacterium* (92.80% of archaea) was mainly detected in the starch acclimated anaerobic sludge. Therefore, the effect of Fe_3O_4 on anaerobic treatment performance was significantly dependent on the organic carbon acclimated microbial communities.

Keywords: Ferroferric oxide; Methanogenesis; Hydrolysis/acidification; Direct interspecies electron transfer

1. Introduction

Anaerobic processes are widely used for the treatment of wastewater with high concentrations of organic carbon. Anaerobic wastewater treatment can contribute to both pollution control and energy recovery, making it one of sustainable technologies for wastewater management. The interspecies electron transfer via H_2 or formate plays an important role in methane (CH_4) production by methanogens. Recently, direct interspecies electron transfer (DIET) has been demonstrated to be another mechanism responsible for electron transfer in anaerobic processes [1]. During DIET, microorganisms possess the ability to exchange electrons through biological electrical connections such as cellular pili. On the other hand, conductive materials, such as granular activated carbon, carbon cloth, carbon nanotube, and ferroferric oxide (Fe_3O_4), can also facilitate DIET for syntrophic CH_4 production [2–6].

Conductive materials function as electron conduits between fermenting bacteria and methanogens, enhancing the CH_4 production rate and shortening the lag phase of CH_4 production. Organic substrates such as ethanol, acetate, butyrate and propionate have been applied to investigate the facilitation of conductive materials on anaerobic treatment

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systems or co-cultures between methanogens and acidogenic bacteria [2,4,6–8]. Wastewater usually contains complicated organic carbons, and their anaerobic treatment requires complex degradation processes. However, complicate organic substrates have been less investigated, and the ability of extracellular electron transfer from other acidogenic bacteria and methanogens remains unknown. Up to date, only *Geobacter*, *Methanosarcina* and *Methanosaeta* have been proposed to be capable of transferring or accepting electrons via DIET, and pili or c-type cytochrome has been shown to be responsible for the extracellular electron transfer [9–11]. Since microorganisms enriched from different types of organic carbons may be diverse, the relationship between DIET and organic carbons requires further investigation.

Fe_3O_4 is the main component of magnetite, and the facilitation of methanogenesis by magnetite has been confirmed in several studies [6,7]. Therefore, in this study, Fe_3O_4 was chosen as the conductive material to examine its effect on the anaerobic treatment of high strength wastewater. Furthermore, the effect of organic substrates, i.e., starch and tryptone, on anaerobic treatment under conditions with the dosage of Fe_3O_4, was investigated. Finally, microbial community of the used anaerobic sludge was also analyzed to examine its effect on DIET.

2. Materials and methods

2.1. Anaerobic sludge acclimation

Anaerobic sludge was taken from two lab-scale anaerobic reactors fed with tryptone and starch, respectively. The reactors had been continuously operated at 35°C with a hydraulic retention time of 48 h and a volumetric chemical oxygen demand (COD) loading rate of 1,500 mg/(L·d). The components of synthetic wastewater were as follows: 290 mg/L NH_4Cl, 100 mg/L $CaCl_2$, 200 mg/L $MgCl_2$, 70 mg/L Na_2HPO_4, 200 mg/L $KHCO_3$ and 1 mL/L trace elements. Trace elements consisted of 1 g/L $FeCl_2{\cdot}4H_2O$, 100 mg/L $CoCl_2{\cdot}6H_2O$, 200 mg/L $NiCl_2{\cdot}6H_2O$, 100 mg/L $MnCl_2{\cdot}4H_2O$, 100 mg/L $NaMoO_4{\cdot}2H_2O$, 100 mg/L H_3BO_3, 100 mg/L $NaWO_4{\cdot}2H_2O$ and 100 mg/L $NaSeO_3$.

2.2. Batch experiments

After the two reactors reached steady state, the following experiments were carried out to examine effects of: (i) the Fe_3O_4 dosage on methanogenesis of tryptone and starch, (ii) the dosed Fe_3O_4 concentrations on methanogenesis of tryptone and starch, (iii) the Fe_3O_4 dosage on hydrolysis and acidification of tryptone and (iv) the Fe_3O_4 dosage on methanogenesis of acetate.

For experiment (i), tryptone or starch was used as the organic carbon, and two groups with (the Fe_3O_4 group) and without (the control group) the dosage of 10 g/L Fe_3O_4 were examined. For experiment (ii), tryptone or starch was used as the organic carbon, and the dosed Fe_3O_4 concentrations of 0, 2.5, 5, 10, 15 and 20 g/L (noted as groups of the control, F2.5, F5, F10, F15 and F20, respectively) were applied. For experiment (iii), tryptone was used as the organic carbon, and two groups with (the Fe_3O_4 group) and without (the control group) the dosage of 10 g/L Fe_3O_4 were carried out. During experiment (iii), 10 mmol/L 2-bromoethanesulfonic acid sodium salt (BES) was dosed to inhibit the activities of methanogens for CH_4 production. For experiment (iv), acetate was used as the solo organic carbon to examine the methanogenesis of acetate only, and two groups with (the Fe_3O_4 group) and without (the control group) the dosage of 10 g/L Fe_3O_4 were carried out.

All batch experiments were carried out with replications in 500 mL batch reactors. The reactors were fed with 200 mL of anaerobic sludge, 300 mL of nutrient solution and 0.5 mL of trace element solution. Before each experiment, nitrogen gas (N_2) was used to remove the oxygen from the headspace of the reactors for 3 min, and then reactors were sealed with rubber stoppers and mixed in an air bath shaker at 170 r/min and 35°C. The suspended solids (SS) and volatile suspended solids (VSS) concentrations in these experiments were around 2.39 ± 0.22 g/L and 2.04±0.21 g/L, respectively. Liquid and gas samples were periodically collected to analyze concentrations of COD, volatile fatty acids (VFAs) and CH_4, respectively.

2.3. Analytical methods

COD, SS and VSS were measured according to standard methods [12]. The liquid samples were filtered through 0.45 μm filter membranes, and then the concentration of soluble COD was measured.

CH_4 was measured by a gas chromatograph (GC-2014, Shimadzu, Japan) equipped with a thermal conductivity detector and a 2-m packed column (Porapak N, Agilent Technologies, USA). The temperatures of injector, detector and column were kept at 90°C, 100°C and 35°C, respectively. Helium gas was used as the carrier gas at a flow rate of 25 mL/min. The modified Gompertz model [13] was used to analyze the kinetic parameters of CH_4 production, which included the maximum CH_4 yield (P_{max}), the maximum CH_4 production rate (R_{max}) and the lag phase time (λ).

VFAs were tested by a gas chromatograph (GC-2014, Shimadzu, Japan) equipped with a flame ionization detector and a capillary column. The carrier gas was N_2 at a flow rate of 50 mL/min, with a split ratio of 15 at a flow rate of 1.1 mL/min in the column and a purge flow rate of 3.0 mL/min. The oven temperature was increased proportionally from 70°C to 200°C within 10 min, and the final holding duration was 2 min. The temperatures of both injector and detector were 240°C. The injected volume of the pre-acidified samples (adjust the pH to <3 with formic acid) was 1 μL.

DNA was extracted from anaerobic sludge in the two reactors fed with tryptone and starch using the PowerSoil DNA extraction kit (Laboratories Inc., CA, USA). After extraction, the DNA sample was amplified by the V4 region of the bacterial 16S rRNA gene and analyzed by high-throughput sequencing using the Illumina Miseq platform [14]. In order to analyze the bacterial diversity of the samples, the sequences obtained were phylogenetically allocated down to the phylum, class, order, family and genus level using the MOTHUR program at a 97% similarity, and a confidence threshold of 95% was set for the taxonomic assignment. The relative abundance of individual taxa within each community was estimated by comparing the number of sequences assigned to a specific taxon and the number of total sequences obtained for that sample.

3. Results and discussion

3.1. Effect of the Fe_3O_4 dosage on methanogenesis of tryptone and starch

Fig. 1 shows the effect of the dosage of Fe_3O_4 on the variation of CH_4 production and acetic acid concentrations during methanogenesis of tryptone and starch. For the tryptone acclimated anaerobic sludge, the dosage of Fe_3O_4 shortened the lag phase of CH_4 production and increased the CH_4 production rate, while the total amount of the produced CH_4 was less affected. Dynamics of CH_4 production were well fitted with the modified Gompertz model (R^2 of 0.999 for the control group and 0.995 for the Fe_3O_4 group). In the control group and the Fe_3O_4 group, the obtained total CH_4 yield was 117.3 and 112.5 mL; the lag phase time was 5.0 and 2.6 h; and the maximum CH_4 production rate was 5.1 and 6.9 mL/h, respectively. Therefore, with the dosage of Fe_3O_4, the lag phase of CH_4 production was shortened by 48.0%, and the maximum CH_4 production rate was increased by 35.2%. The VFAs consumption rate, especially acetic acid, was faster by adding Fe_3O_4. For example, the acetic acid concentration at 15th hour in the control group and the Fe_3O_4 group was 95.8 and 9.8 mg/L, respectively.

For the starch acclimated anaerobic sludge, the dosage of Fe_3O_4 had little effect on the CH_4 production during the initial 20 h, and thereafter a high production occurred in the Fe_3O_4 group. Simulated with the modified Gompertz model, during the experiment, the maximum CH_4 production rate in the control group and the Fe_3O_4 group was 2.8 and 3.3 mL/h, and the lag phase time was 5.4 and 6.4 h, respectively. Therefore, with the dosage of Fe_3O_4, the maximum CH_4 production rate was increased by 17.9%, and the lag phase of CH_4 production was also increased by 18.5%. For VFAs, the highest acetic acid concentration in the control group and the Fe_3O_4 group was 103.1 and 227.6 mg/L, respectively. This showed that the dosage of Fe_3O_4 enhanced the production of acetic acid.

Fig. 2 shows the effect of dosed Fe_3O_4 concentrations on the CH_4 production and acetic acid concentrations during methanogenesis of tryptone and starch. For the tryptone acclimated anaerobic sludge, fitted with the modified Gompertz model, the lag phase in the control, F2.5, F5, F10, F15 and F20 groups was 9.2, 7.5, 7.0, 6.6, 5.5 and 4.3 h, respectively, and the maximum CH_4 production rate was 4.3, 5.0, 5.2, 5.5, 5.5 and 4.9 mL/h, respectively. Therefore, with increasing the Fe_3O_4 concentration, the lag phase was shortened, and the maximum CH_4 production rate increased except at the dosed Fe_3O_4 concentration of 20 g/L. The obtained optimal dosed Fe_3O_4 concentration for enhancing CH_4 production was around 10 g/L.

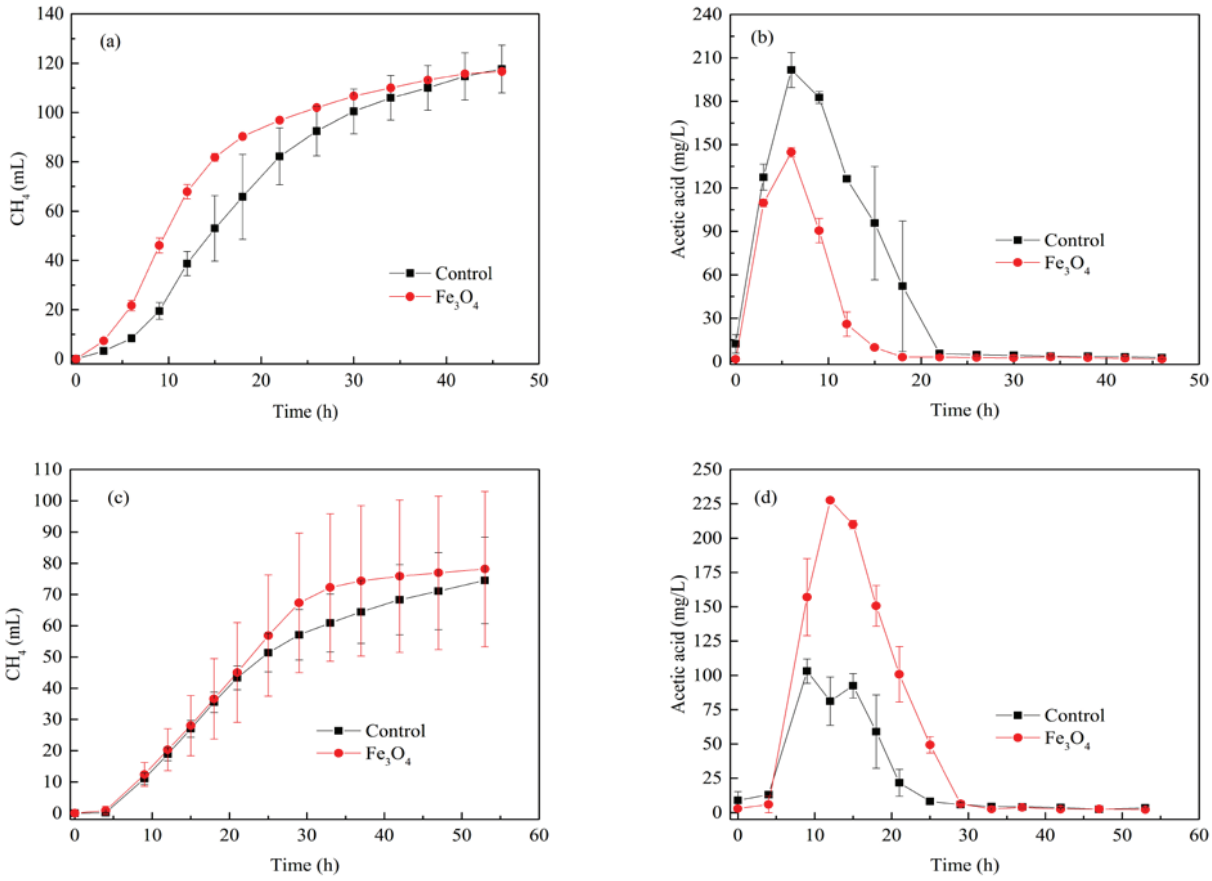

Fig. 1. Methane production (a) and acetic acid production (b) using tryptone as the organic carbon, and methane production (c) and acetic acid production (d) using starch as the organic carbon.

For the starch acclimated anaerobic sludge, the lag phase of the control, F2.5, F5, F10, F15 and F20 groups was 4.3, 4.9, 4.9, 6.4, 4.8 and 5.0 h, respectively, and the maximum CH_4 production rate was 3.0, 3.4, 3.5, 3.0, 2.5 and 1.8 mL/h, respectively. Therefore, the dosage of Fe_3O_4 elongated the lag phase for CH_4 production, and the maximum CH_4 production rate was only increased by 13.3% in the F2.5 group and 16.7% in the F5 group. On the other hand, the highest acetic acid concentration in the control, F2.5, F5, F10, F15 and F20 groups was 88.3, 92.4, 99.2, 152.7, 146.5 and 122.8 mg/L, respectively. Thus, the dosage of Fe_3O_4 increased the maximum acetic acid concentration to different levels. The increased accumulation of VFAs was probably due to the low efficiency of CH_4 production, according to the prolonged lag phase.

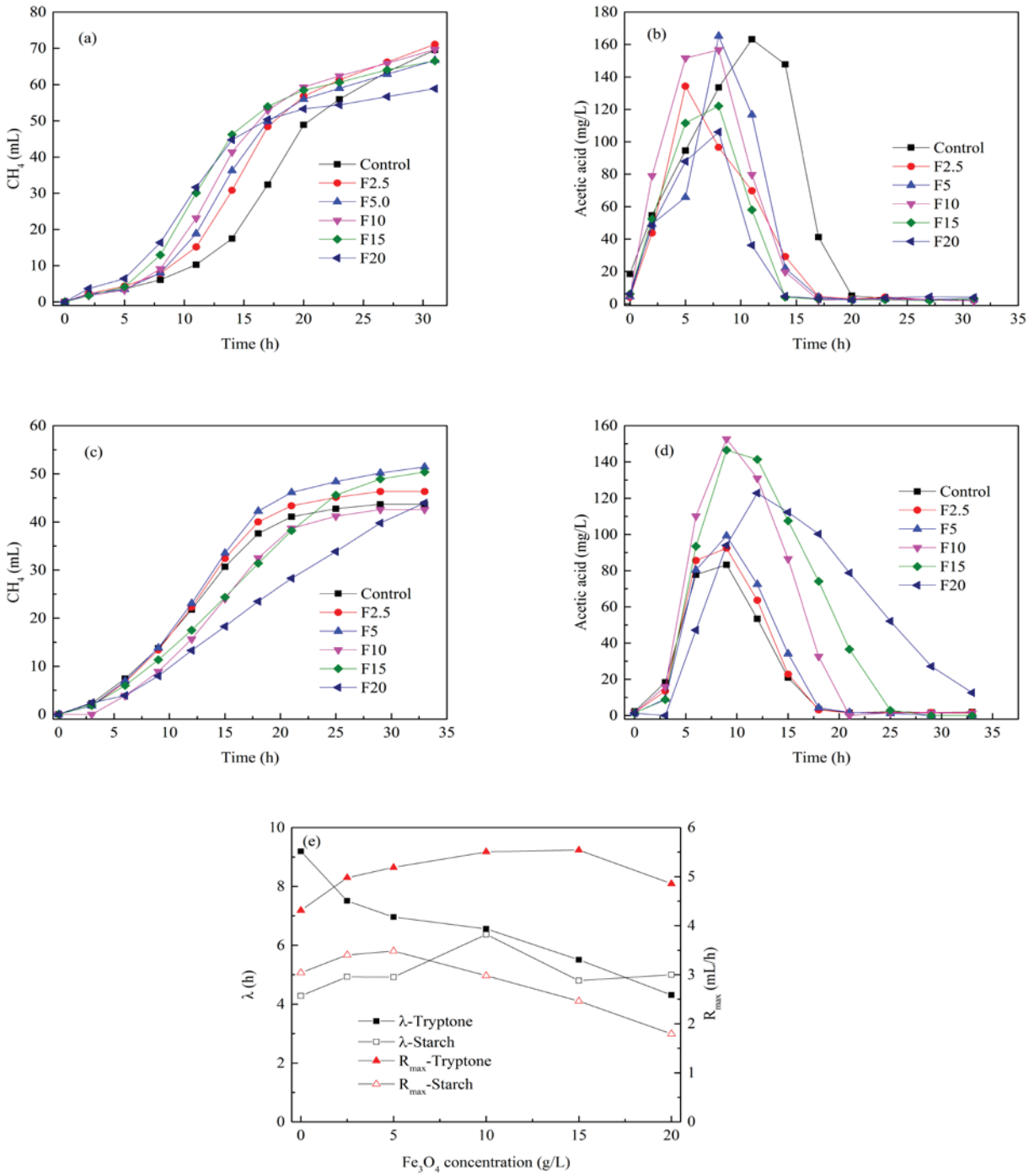

Fig. 2. Methane production (a) and acetic acid production (b) with varying Fe_3O_4 concentrations using tryptone as the organic carbon, and methane production (c) and acetic acid production (d) with varying Fe_3O_4 concentrations using starch as the organic carbon and (e) the relation between the Fe_3O_4 concentrations and both λ and R_{max}.

Fig. 2(e) shows the relationship between the Fe_3O_4 concentration and the lag phase and the maximum CH_4 production rate. For the tryptone acclimated anaerobic sludge, with increasing Fe_3O_4 concentrations, the lag phase was shortened by 18.3%–53.1%, and the maximum methane production rate also increased obviously until to the Fe_3O_4 concentration of 10 g/L (no further increase from 10 to 20 g/L). For the starch acclimated anaerobic sludge, the maximum CH_4 production rate was increased only at the Fe_3O_4 concentrations of 2.5 and 5 g/L, while the lag phases were all longer than the control group with the dosage of various concentrations of Fe_3O_4.

From above results, it confirmed that the dosage of Fe_3O_4 enhanced CH_4 production (increasing the production rate and shortening the lag phase) for the tryptone acclimated anaerobic sludge, while less was observed for the starch acclimated anaerobic sludge. Therefore, in the following batch experiments, effects of the dosage of Fe_3O_4 on methanogenesis and hydrolysis/acidification processes were carried out only for the tryptone acclimated anaerobic sludge.

3.2. Effects of the dosage of Fe_3O_4 on hydrolysis/acidification of tryptone and methanogenesis of acetate

To further evaluate the effect of Fe_3O_4 on the hydrolysis and acidification of tryptone, 10 mmol/L BES was added to inhibit methanogen activities. During hydrolysis and acidification of tryptone, no CH_4 was produced, and propionic acid, butyric acid and acetic acid were accumulated gradually. The final VFAs concentration of the control group and the Fe_3O_4 group was 626.2 and 629.7 mg/L, respectively. The VFAs to COD ratio was 69.0% and 75.1%, respectively. Fig. 3 shows that the control group and the Fe_3O_4 group had similar trend for VFAs production, indicating that Fe_3O_4 might not facilitate the hydrolysis/acidification of tryptone.

To examine the effect of dosage of Fe_3O_4 on methanogenesis of acetate without the participation of hydrolysis and acidification, sodium acetate was used as the organic carbon instead of tryptone. Without hydrolysis and acidification, the dosage of Fe_3O_4 seemed to hinder the activities of methanogens (Fig. 4). According to the modified Gompertz model, the lag phase time of the control group and the Fe_3O_4 group was 17.9 and 19.6 h; the maximum CH_4 production rate was 6.4 and 1.7 mL/h; and the ultimate CH_4 yield was 72.6 and 67.9 mL, respectively. Therefore, when sodium acetate was used as the organic carbon, the maximum CH_4 production rate was decreased by 73.4%, and the lag time was delayed by 9.5%.

The dosage of Fe_3O_4 did not enhance the hydrolysis and acidification of tryptone or methanogenesis of acetate. These results showed that the acceleration of CH_4 production by the dosage of Fe_3O_4 only occurred when the interspecies electron transfer between fermenting bacteria and methanogens was enhanced. The conductive property of Fe_3O_4 might be the key factor accelerating DIET for syntrophic CH_4 production [4,15], and a high Fe_3O_4 concentration provided a better conductive condition leading to a high acceleration. When sodium acetate was used as the organic carbon, interspecies electron transfer was absent. Under this condition, the dosage of Fe_3O_4 might inhibit the transfer efficiency between methanogens and sodium acetate, leading to the decreased maximum CH_4 production rate and the increased lag phase.

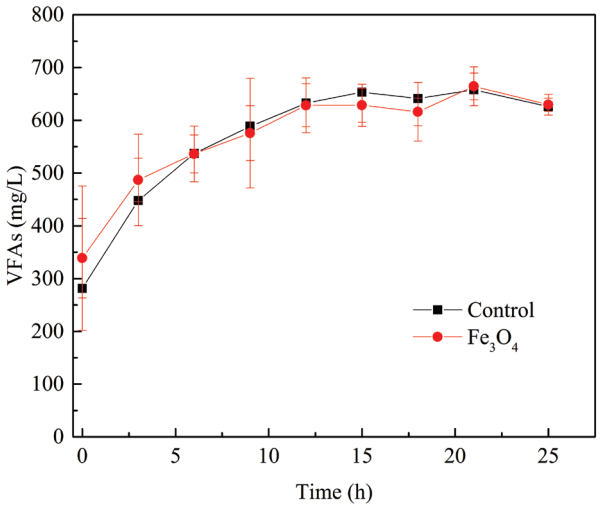

Fig. 3. VFA production during hydrolysis and acidification of tryptone for the tryptone acclimated anaerobic sludge.

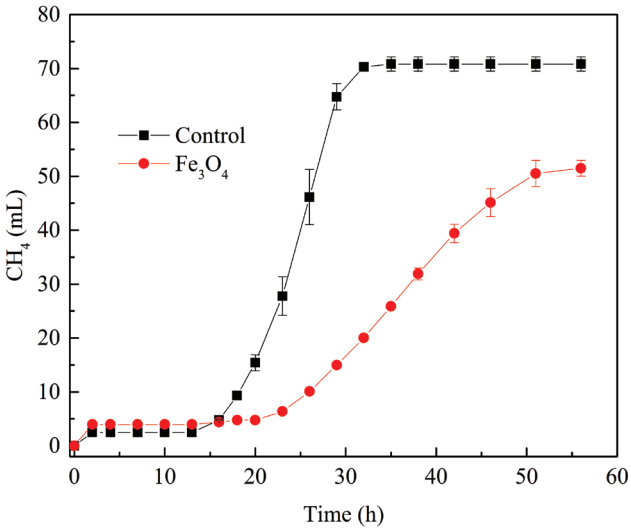

Fig. 4. Methane production using acetate as the organic carbon for the tryptone acclimated anaerobic sludge.

3.3. Microbial community structure analysis with high-throughput sequencing

Fig. 5 shows the microbial communities for the anaerobic sludge acclimated with tryptone or starch. At the phylum level, *Bacteroidetes* and *Firmicutes* were dominant in the tryptone acclimated anaerobic sludge, with the proportion of 47.9% and 34.9%, respectively. *Proteobacteria*, *Bacteroidetes* and *Firmicutes* were predominant in the starch acclimated anaerobic sludge, with the proportion of 41.1%, 32.2% and 14.6%, respectively. At the genus level, *Methanosarcina*, *Clostridium*, *Syntrophomonas* and *Methanosaeta* were detected species in the tryptone acclimated anaerobic sludge, with the proportion of 4.1%, 3.4%, 1.9% and 1.2%, respectively. *Aeromonas*, *Azonexus*, *Thauera*, *Acinetobacter*, *Methanobacterium* were detected in the starch acclimated anaerobic sludge, with the proportion of 15.6%, 6.6%, 4.7%, 3.8% and 3.7%, respectively.

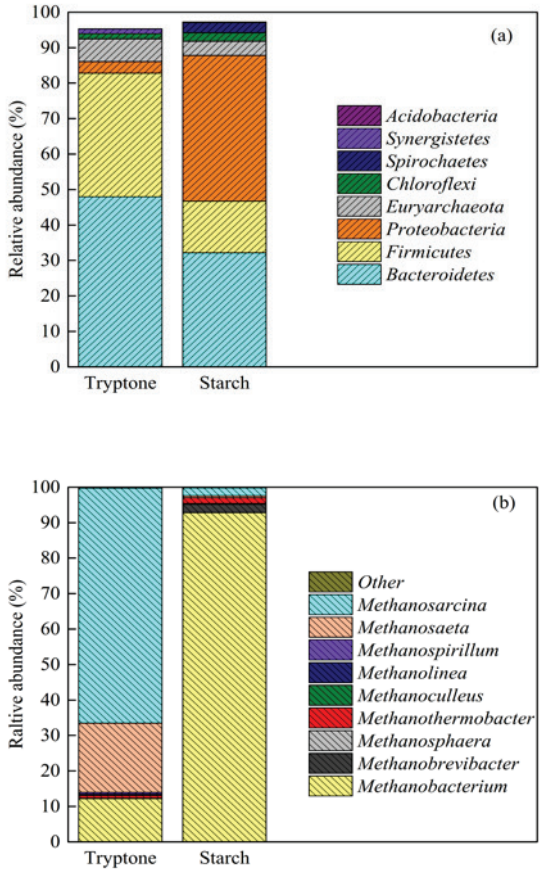

Fig. 5. Relative abundance of microbial communities at the phylum level (a) and archaeal community structure at the genus level for the anaerobic sludge acclimated with tryptone or starch (b), respectively.

The detected methanogens in the tryptone acclimated anaerobic sludge were *Methanosarcina*, *Methanosaeta* and *Methanobacterium*, with the proportion of 66.3%, 19.6% and 12.2%, respectively. In the starch acclimated anaerobic sludge, *Methanobacterium* was the dominant methanogen, with the proportion of 92.8%.

Many organic carbons, such as ethanol [2,3,9], glucose [16], butyrate [4], propionate [7,15] and benzoate [6], have been applied to examine the facilitation of conductive materials on the methanogenesis process. All results have confirmed the acceleration of CH_4 production by dosing conductive materials. However, in our study, the dosage of Fe_3O_4 enhanced the CH_4 production significantly for the tryptone acclimated anaerobic sludge, while less was observed in the starch acclimated anaerobic sludge. Therefore, organic carbon seemed to affect the performance of conductive materials on CH_4 production. In the tryptone acclimated anaerobic sludge, *Methanosarcina*, *Methanosaeta* and *Methanobacterium* were dominant methanogens. *Methanosarcina* and *Methanosaeta* were shown to have the ability of DIET [9,10], and these two species occupied over 85.9% of the total archaea. Nevertheless, in the starch acclimated anaerobic sludge, *Methanosarcina* and *Methanosaeta* only accounted for 2.8% of the total archaea, while *Methanobacterium*, a typical H_2-utlizing methanogen, accounted for 92.8% of the total archaea. Whether *Methanobacterium* could involve in DIET is still controversial [4,10,17]. According to the above results, *Methanobacterium* might not participate in DIET. Therefore, the different proportions and types of methanogens might explain why the CH_4 production was enhanced significantly by dosing Fe_3O_4 for the tryptone acclimated anaerobic sludge, while less was observed for the starch acclimated anaerobic sludge.

Due to the DIET ability of *Geobacter*, which can metabolize ethanol to acetate, many studies have used co-cultures of *Geobacter* and methanogens to testify the effect of conductive materials enhancing DIET [2,3,9]. However, in the tryptone acclimated anaerobic sludge, no *Geobacter* was detected, and in the starch acclimated anaerobic sludge, the relative abundance of *Geobacter* accounted for only 0.35%. These results were reasonable because *Geobacter* mainly enriched with ethanol as the organic carbon. In the tryptone acclimated anaerobic sludge, at the phylum level, *Bacteroidetes* and *Firmicutes* accounted for 47.9% and 34.9%, respectively. *Bacteroidetes* and *Firmicutes* might play important roles in protein and starch degradation [18]. In the starch acclimated anaerobic sludge, the fermenting bacteria *Proteobacteria* accounted for 41.1%, and followed by *Bacteroidetes* and *Firmicutes*, accounting for 32.2% and 14.6%, respectively. Possibly, microorganisms other than *Geobacter* might participate in DIET. Cruz et al. [15] believed that magnetite particles (with the main component of Fe_3O_4) facilitated DIET between acetogens and methanogens, which further promoted the propionate consumption and the CH_4 production. Li et al. [5] dosed single-walled carbon nanotubes in anaerobic digesters and enhanced the CH_4 production and the sucrose decomposition, despite no *Geobacter* was detected. Zhao et al. [19] reported that *Syntrophomonas* species were enriched in two carbon-felt reactors and proposed that *Syntrophomonas* species were likely to participate in DIET for the sludge decomposition and the CH_4 production. In the present study, 15.0% of the genera in the tryptone acclimated anaerobic sludge was *Syntrophomonas* within the *Firmicutes* phylum, while the proportion was only 1.0% in the starch acclimated anaerobic sludge. Therefore, it could be further confirmed that some species other than *Geobacter* such as *Syntrophomonas* might also transfer electrons via DIET. Microbial communities of anaerobic sludge could be shaped by different organic carbons, showing different responses when conductive materials were dosed. Furthermore, it should be noted that the present results were mainly based on the short-term dosage of Fe_3O_4 and the effect of long-term dosage of conductive materials on methanogenesis deserves further investigation. For practical application, the dosage of Fe_3O_4 in anaerobic processes would enhance the system performance for treating protein-based organic carbons.

4. Conclusions

The dosage of Fe_3O_4 accelerated methane production significantly for the tryptone acclimated anaerobic sludge. This acceleration during the batch dosage of Fe_3O_4 only occurred when the interspecies electron transfer between fermenting bacteria and methanogens. The conductive property of Fe_3O_4

might be the reason for the acceleration of DIET for syntrophic CH_4 production. *Methanosarcina* and *Methanosaeta* were dominant methanogens in the tryptone acclimated anaerobic sludge, while *Methanobacterium* was the dominant methanogen in the starch acclimated anaerobic sludge. Organic carbon affected the acclimated microbial communities, leading to different performance when dosing conductive materials.

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