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Chitosan-Fe_3O_4 composites enhance anaerobic digestion of liquor wastewater under acidic stress

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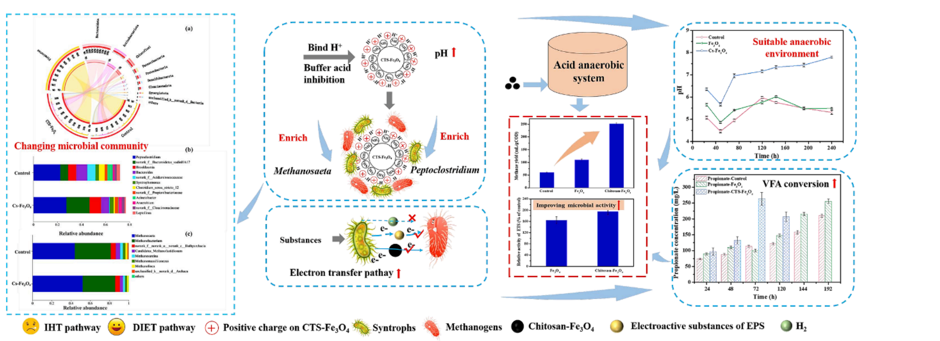
HIGHLIGHTS

- Acid inhibition was alleviated by the addition of chitosan-Fe_3O_4.
- Chitosan-Fe_3O_4 promoted the utilization of organic acids under acidic pressure.
- Chitosan-Fe_3O_4 supplemented could enhance the electron transfer activity.
- Chitosan-Fe_3O_4 enriched microbes related to direct interspecies electron transfer.

GRAPHICAL ABSTRACT



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ABSTRACT

Acid stress in the anaerobic digestion process of liquor wastewater leads to low anaerobic treatment efficiency. Herein, chitosan-Fe_3O_4 was prepared, and its effects on anaerobic digestion processes under acid stress were studied. Results showed that chitosan-Fe_3O_4 increased the methanogenesis rate of anaerobic digestion of acidic liquor wastewater by 1.5–2.3 times and accelerated the restoration of acidified anaerobic systems. The analysis of sludge characteristics showed that chitosan-Fe_3O_4 promoted the secretion of proteins and humic substances in extracellular polymeric substances and increased the electron transfer activity of the system by 71.4%. Microbial community analysis indicated that chitosan-Fe_3O_4 enriched the abundance of *Peptoclostridium*, and *Methanosaeta* participated in direct interspecies electron transfer. Chitosan-Fe_3O_4 could promote the direct interspecies electron transfer pathway to maintain stable methanogenesis. These methods and results regarding the use of chitosan-Fe_3O_4 could be referred to for improving the efficiency of anaerobic digestion of high concentration organic wastewater under acid inhibition.

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1. Introduction

The liquor industry has grown rapidly in recent years, and as one of the characteristic industries in China, it has brought significant economic benefits. According to the data from the China Liquor Industry Association, China produced 7.1 billion liters of liquor in 2021. However, the winemaking process can produce liquor wastewater, with more than 15.0 L of liquor wastewater produced in the production of 1.0 L of liquor (Luo et al., 2018). Liquor wastewater mainly contains polysaccharides, organic acids, ethanol, and other organic compounds, so its chemical oxygen demand (COD) is high (greater than 35 g/L), and its pH is about 3.0–5.0 (Luo et al., 2018). Therefore, improper liquor wastewater treatment will lead to river pollution and vegetation damage (Pant & Adholeya, 2007; Tan et al., 2022). Anaerobic digestion (AD) technology has the benefits of high reliability, low cost, and bioenergy recovery (Ambaye et al., 2021), which can be used for liquor wastewater treatment. However, the performance of AD is easily affected by intermediate products, toxic substances, adverse environments, and other factors (Huang et al.,2022; Liu et al., 2021b; Tan et al., 2021). For liquor wastewater with pH 3.0–5.0, the acidic environment will inhibit the activity of methanogens, resulting in volatile fatty acids (VFA) accumulation and anaerobic systems collapse (Wu et al., 2020). The stability of AD can be maintained by adding alkaline chemicals (Zhai et al., 2015), but the accumulation of VFA may occur again when alkaline chemicals are consumed. Therefore, it is urgent to develop a more efficient, stable, and economical technology to improve the performance of AD and recover bioenergy from acid liquor wastewater.

Recently, conductive materials (CMs) such as biochar, carbon cloth, zero-valent iron, and magnetite have been widely chosen to improve AD performance under adverse conditions (Wambugu et al., 2019; Zhou et al.,2021). Traditional interspecies hydrogen transfer (IHT) is easily inhibited in acidic environments, but the introduction of CMs constructs direct interspecies electron transfer (DIET) (Zhao et al., 2017b). Promoting DIET in anaerobic systems can stabilize the methanogenesis of carbohydrates, and carbon cloth and graphene can promote DIET to alleviate acid inhibition (Wu et al., 2020; Zhao et al., 2017b). Metal-based conductive materials are more prominent in promoting fatty acid conversion than carbon-based conductive materials (Zhao et al., 2017a). However, the study of adding metal-based CMs to enhance AD performance under acidic stress has yet to be reported.

Fe_3O_4 has attracted increasing attention due to its retrievability and higher efficiency in accelerating the conversion of fatty acids. However, there are still some problems in applying Fe_3O_4 in the AD system. Firstly, the massive dissolution of metal materials is a non-negligible issue. Li et al. (2022) found that the loss of Fe_3O_4 was close to 50.0% after the AD experiment, and the methane yield in the Fe_3O_4 reactor did not significantly improve during the second AD experiment. Domrongpokkaphan et al. (2021) also found that the dissolved iron concentration in the reactor reached about 130.0 mg/L when zero-valent iron was added to the AD process of acid palm oil plant wastewater. Secondly, the surface of Fe_3O_4 has poor microbial adhesion (Su et al., 2020). To achieve the broad application of Fe_3O_4 in an anaerobic system, Fe_3O_4 can be modified by other materials. Chitosan (CTS) is a low-cost biopolymer carrier with good biocompatibility and metal stability (Lan et al., 2022). Besides, CTS contains many amino groups ($-NH_2$) and has a positive surface charge, thus having a high microbial affinity, and has been used to promote the formation of anaerobic granular sludge (Torres et al., 2018). Studies have found that iron-loaded chitosan can effectively improve the organic load of the anaerobic system, promote the formation of granular sludge, and improve methanogenesis performance (Wang et al., 2019; Zhang et al., 2020a). Therefore, Fe_3O_4 was modified by CTS to enhance the stability and biocompatibility of Fe_3O_4 in this study.

Currently, few studies have explored the roles of chitosan-Fe_3O_4 composite material (CTS-Fe_3O_4) in the AD process, especially in treating liquor wastewater under acid stress. Thus, chitosan-Fe_3O_4 composites were added to the AD system under acid pressure in this study. The role of CTS-Fe_3O_4 in AD processes of acid liquor wastewater was explored, and the effectiveness of CTS-Fe_3O_4 on the restoration of acid system was evaluated. Moreover, the possible mechanism through microbial communities and sludge characteristics were analyzed.

2. Materials and methods

2.1. Substrate and seed sludge

Seed sludge was collected from the anaerobic tank of the sewage treatment plant of Guozhen Environmental Protection Technology Co., Ltd in Changsha, China. After precipitation, the sludge was passed through a 200 mesh screen to remove impurities. Before the experiment, the collected wastewater was filtered through a mesh (1.0–2.0 mm) and stored in a refrigerator at 4 °C. To obtain highly active inoculum, the pretreated sludge was fed into a laboratory-scale semi-continuous bioreactor, and the substrate was liquor wastewater containing 1000–6000 mg/L COD. The inoculum was obtained when the COD removal rate of liquor wastewater was stable at more than 85.0%, and the sludge maintained high methanogenic activity (see supplementary materials). The primary properties of the sludge and the actual liquor wastewater were determined after collecting the seed sludge and liquor wastewater (Table 1). The volatile suspended solids (VSS) concentration of the inoculum was 35.2 ± 3.2 g/L.

2.2. Synthesis of Fe_3O_4 and chitosan-Fe_3O_4 composites

As previously described, nano-sized Fe_3O_4 was synthesized by the co-precipitation method with minor modifications (Kang et al., 1998). Chitosan-Fe_3O_4 composite material (CTS-Fe_3O_4) was prepared by the in-situ co-precipitation method according to Li et al. (2020) with some modifications. In short, 6.0 g chitosan was added into 200 mL Fe^{3+}/Fe^{2+} mixed solution (Fe^{3+}:Fe^{2+} = 2:1), then 2 mL acetic acid was added, and mechanical mixing for about 60 min. Finally, an injection pump pumped the hybrid solution into a 1.5 mol/L sodium hydroxide solution. After standing in sodium hydroxide solution for 6 h, the gel beads were washed with deionized water and dried in a vacuum oven at 60°C for 24 h. The preparation method of chitosan particles is the same as that of CTS-Fe_3O_4, without adding iron ions.

2.3. Batch experiment design

The experiments were performed in serum bottles with a working volume of 150 mL. The experimental design of batch experiments is shown in Table 2. Previous studies have shown that adding 0.4–2.5 g/g VSS of Fe_3O_4 composites can improve AD performance (Liu et al., 2021a; Su et al., 2020). Therefore, in this study, we investigated the effects of 0, 0.5, 1.5, and 2.0 g/g VSS CTS-Fe_3O_4 on AD of liquor wastewater in the first batch experiment. Besides, to investigate the contribution of chitosan to methane production, one group of the anaerobic system used chitosan microspheres as substrate and another group with only

Table 1
Characteristics of liquor wastewater and inoculum.

Parameter	liquor wastewater	Inoculum
pH	4.0 ± 0.2	7.0 ± 0.1
Total suspended solids (TSS, g/L)	28.2 ± 0.3	59.2 ± 4.8
Volatile suspended solids (VSS, g/L)	22.5 ± 0.3	35.2 ± 3.2
Total COD (TCOD, g/L)	62.5 ± 1.5	
Dissolved COD (SCOD, g/L)	41.5 ± 0.2	
Total nitrogen (TN, mg/L)	2006.0 ± 144.0	
Total phosphorus (TP, mg/L)	380.4 ± 24.0	
NH_4^+-N (mg/L)	620.3 ± 16.3	
SO_4^{2-} (mg/L)	510.0 ± 10.0	

Notes: Each sample was analyzed in triplicate.

Table 2
Experimental set-up of batch experiments.

Experiment	Additive	COD g/L	Inoculum mL	Temperature °C	Volume mL
Experiment 1	/	/	21	35.0 ± 1.0	150
	CTS	/	21	35.0 ± 1.0	150
	(0, 2.5, 5.0, 7.5, 10.0 g/L) $CTS-Fe_3O_4$	5.0 ± 0.3	21	35.0 ± 1.0	150
Experiment 2	/	10.0 ± 1.1	42	35.0 ± 1.0	150
	5.0 g/L $CTS-Fe_3O_4$	10.0 ± 1.1	42	35.0 ± 1.0	150
Experiment 3	/	10.0 ± 1.1	42	35.0 ± 1.0	150
	5.0 g/L Fe_3O_4	10.0 ± 1.1	42	35.0 ± 1.0	150
	5.0 g/L $CTS-Fe_3O_4$	10.0 ± 1.1	42	35.0 ± 1.0	150

Notes: Each experiment was conducted in triplicate.

inoculum and distilled water as a blank control. Based on the results obtained, adding 0.5 g/g VSS $CTS-Fe_3O_4$ had the most obvious effect on the AD performance of liquor wastewater. As shown by the AD performance of acid liquor wastewater (see supplementary materials), when the pH of liquor wastewater was less than 5.0, the COD removal efficiency and methane yield decreased significantly. Therefore, the roles of $CTS-Fe_3O_4$ in the AD process of acid liquor wastewater were investigated in the second experiment. The pH of liquor wastewater in anaerobic systems was selected as 5.0. Considering that the anaerobic system is prone to acidification in the AD of acidic wastewater, the effects of $CTS-Fe_3O_4$ on the acidified anaerobic system were further investigated. The experiment was divided into the acid inhibition stage and the restoration stage. In the acid inhibition stage, the pH of each reactor was adjusted to 5.0. The pH of the control and Fe_3O_4 reactor was adjusted to 7.0 to create a neutral environment during the restoration stage, and the $CTS-Fe_3O_4$ reactor was not treated. All serum bottles were flushed with nitrogen to keep the reaction system anaerobic (Wang et al., 2018). Subsequently, the bottles were placed in a shaker incubator at a speed of 120 rpm and a temperature of 35.0 ± 1.0°C. Each group experiment was conducted in triplicate. The changes in biogas, COD, pH and VFA were measured during the experiment, and the sludge samples were analyzed afterward.

2.4. Analytical methods

For conventional indicators, the total suspended solids, VSS, COD, total nitrogen, ammonia nitrogen, total phosphorus, and SO_4^{2-} were determined by standard methods (APHA, 2005). Biogas components (CO_2, H_2, and CH_4) were determined by a gas chromatograph (GC112A, INESA, China), and individual VFA (acetate, propionate, butyrate, and valerate) were analyzed by a gas chromatograph (GC-2010, Shimadzu, Japan). For sludge properties, the concentrations of soluble protein, polysaccharide, and humic acid were also detected by standard methods. The determination of microbial electron transfer system (ETS) activity in anaerobic sludge was done concerning the previous study (Zhang et al., 2018). Besides, the dissolved organic matter (DOM) in the extracellular polymeric substances (EPS) of anaerobic sludge was qualitatively analyzed by a fluorescence spectrophotometer (Hitachi, FL4500, Japan). The main EPS analyzed were loosely bound extracellular polymeric substances (LB-EPS) and tightly bound extracellular polymeric substances (TB-EPS), and this study extracted EPS from sludge by thermal treatment method (Chen et al., 2018). Each sample in the research was analyzed in triplicate, and the results were displayed as mean ± standard deviation. Analysis of Variance (ANOVA) was performed to identify the statistical significance using SPSS 22.0, and p greater than 0.05 was considered statistically insignificant.

2.5. Microbial community analysis

The microbial community in each reactor was analyzed using high-throughput 16S rRNA gene sequencing. After the experiment, the sludge samples obtained from reactors were analyzed by high-throughput sequencing. The 16S rRNA gene of bacteria and archaea was amplified by polymerase chain reaction (PCR). The bacterial 16S rRNA gene was amplified using primers 338F and 806R, and the archaeal 16S rRNA gene was amplified using primers 524F10extF and Arch958RmodR (Liu et al., 2022). All PCR reactions for each sample were conducted in triplicate. The microbial sequencing data analysis in this study was performed on the online platform of the Majorbio Cloud Platform.

3. Results and discussion

3.1. Effects of chitosan-Fe_3O_4 composites on anaerobic digestion of liquor wastewater

To explore the optimal addition amount of $CTS-Fe_3O_4$, the effects of different concentrations of $CTS-Fe_3O_4$ on the AD performance of liquor wastewater were investigated. Compared with no $CTS-Fe_3O_4$, adding 0.5 g/g VSS $CTS-Fe_3O_4$ increased methane production by 16.2%, and the addition of 1.0 and 1.5 g/g VSS $CTS-Fe_3O_4$ increased methane production by 3.8% and 6.1%, respectively (see supplementary materials). The obvious difference in methane production among these groups demonstrated that the dosage of $CTS-Fe_3O_4$ affected anaerobic metabolism. With the increase of $CTS-Fe_3O_4$ dosage, the methanogenesis performance gradually decreased, and when the dose was 2.0 g/g VSS, the methane yield decreased by 5.7%. This finding is similar to other research showing that a high concentration of iron-based composites might inhibit methanogenesis (Zhu et al., 2021). Three aspects mainly cause this phenomenon: First, the non-selective adsorption of organic matter by the high dose of $CTS-Fe_3O_4$ resulted in the decrease of substrate available to microorganisms. Second, the Fe^{2+} in the solution may combine with the protein to form non-biodegradable organic matter, leading to a decrease in substrate utilization efficiency by anaerobic microorganisms (Dai et al., 2017); Third, excess Fe_3O_4 particles are toxic to microbial cells and can inhibit methanogenesis (Zhu et al., 2021). Therefore, Fe_3O_4 in high doses of $CTS-Fe_3O_4$ might also cause damage to anaerobic microorganisms, resulting in lower methane production. Considering the operating cost, the optimal dosage of $CTS-Fe_3O_4$ was 0.5 g/g VSS, which was used in subsequent studies.

3.2. Effects of chitosan-Fe_3O_4 composites on anaerobic digestion under acid stress

The effects of $CTS-Fe_3O_4$ (0.5 g/g VSS) on the AD of acidic liquor wastewater were illustrated in Fig. 1. During the first acidic shock, the control reactor had a low methanogenesis rate and a long methanogenesis lag period, indicating that the methanogenic process in the control group began to be inhibited. According to the modified Gompertz model, adding $CTS-Fe_3O_4$ increased the methanogenesis rate by 49.7% and shortened the lag time by 30.4% (Table 3). However, adding $CTS-Fe_3O_4$ had no significant effect on cumulative methane production. This phenomenon was similar to the research results of Luo et al. (2015). At the initial stage of the experiment, the pH in the control reactor was between 5.5 and 6.0 (Fig. 1f). The pH of the anaerobic system below 5.3 is not conducive to methanogenesis (Wu et al., 2010). Hence, the activity of methanogenic archaea in the reactor was not completely inhibited. With the utilization of organic acids in the anaerobic system, the system's pH gradually returned to a normal level. Therefore, there was no significant difference in cumulative methane production between the $CTS-Fe_3O_4$ and the control groups. However, the pH of the $CTS-Fe_3O_4$ reactor was maintained at a normal level throughout the experiment, and the activity of methanogens in the $CTS-Fe_3O_4$ reactor

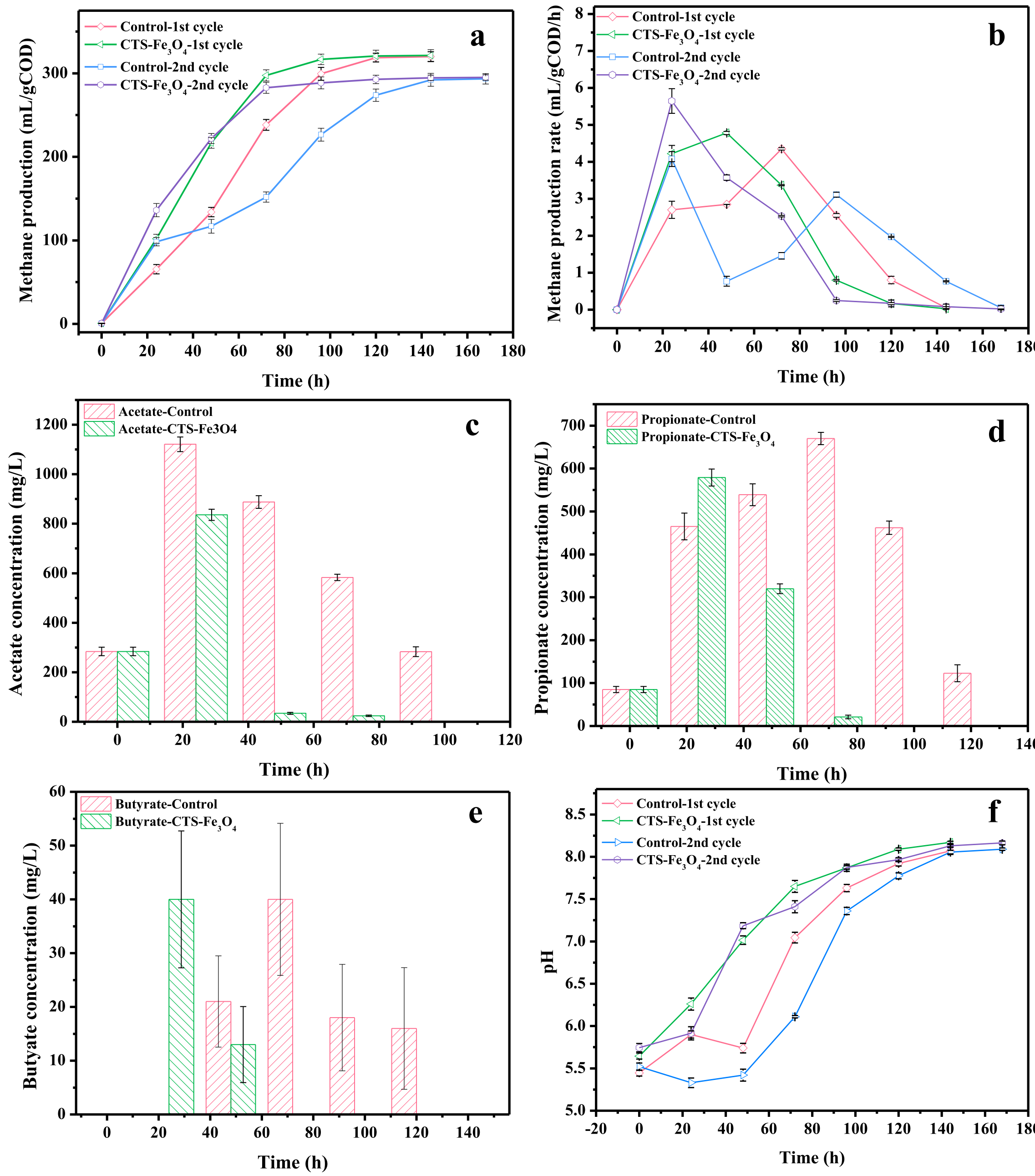

Fig. 1. Effects of chitosan-Fe_3O_4 addition on anaerobic digestion performance of acid liquor wastewater. (a) Methane production; (b) Methane production rate; (c) Acetate under second cycle; (d) Propionate; (e) Butyrate; (f) pH.

was high. Besides, the stability of CTS-Fe_3O_4 in the AD process of acid liquor wastewater was studied by secondary cycle. From Fig. 1a, in the second cycle experiments, the methane production performance of the reactor was inhibited. CTS-Fe_3O_4 could also improve the AD performance of acid liquor wastewater. The methane production rate in the CTS-Fe_3O_4 reactor increased by 1.3 times, and the methanogenesis lag time decreased by 46.4%. The results of the second cycle experiment demonstrated that CTS-Fe_3O_4 could continuously and efficiently enhance the AD performance of acid liquor wastewater.

To further understand the effects of CTS-Fe_3O_4 in the AD process of acid liquor wastewater, the changes in VFA with time were observed. Fig. 1c shows that the acetate concentration in the CTS-Fe_3O_4 reactor almost reached its maximum at 24 h, then degraded at 72 h. However, the VFA concentration in the control group reached the maximum at 48 h, and the VFA concentration was still 1598.0 mg/L at 72 h. Besides, adding CTS-Fe_3O_4 significantly promoted the degradation of propionic acid and butyric acid, which was similar to the results of Viggi et al. (2014). The degradation efficiency of propionic acid and butyric acid in the control group was low, mainly because the oxidation efficiency of propionic acid and butyric acid decreased under acid pressure (Wu et al., 2020). The accumulation of propionic acid and butyric acid during anaerobic digestion leads to decreased methanogenic performance. Fig. 1f shows that the pH of the reactor system with CTS-Fe_3O_4 was stable between 6.5 and 7.5, while the pH of the control reactor was only 5.5. Therefore, the activity of methanogens in the reactor is inhibited, resulting in a low methanogenesis rate and a long methanogenesis lag period in the control reactor. These findings suggested that adding CTS-Fe_3O_4 was beneficial to increase the pH of the anaerobic system, thus reducing the acidic inhibition effect on methanogens and improving the AD performance of acidic liquor wastewater. These results suggested that adding CTS-Fe_3O_4 could promote the utilization of VFA and keep the system in a suitable pH range.

Table 3
Effects of chitosan-Fe_3O_4 on methanogenesis kinetics using modified Gompertz model.

Reactors	$P_{measured}$ (mL/gCOD)	Kinetic parameters			
		P (mL/gCOD)	R_{max} (mL/gCOD/h)	λ (h)	R^2
1st Control[a]	319.97 ± 6.98	326.34 ± 22.73	4.83 ± 0.47	16.56 ± 2.64	0.987
1st CTS-Fe_3O_4 [a]	321.36 ± 5.98	316.58 ± 10.13	7.23 ± 0.54	11.52 ± 1.20	0.996
2nd Control[a]	293.43 ± 6.31	286.31 ± 40.34	3.56 ± 0.62	16.56 ± 5.76	0.943
2nd CTS-Fe_3O_4[a]	295.05 ± 3.28	286.78 ± 8.40	8.30 ± 0.75	8.88 ± 1.20	0.995
Control[b]	60.60 ± 1.49	74.67 ± 3.13	0.36 ± 0.01	41.42 ± 2.19	0.999
Fe_3O_4[b]	109.94 ± 4.73	144.13 ± 13.04	0.61 ± 0.03	41.39 ± 3.78	0.995
CTS-Fe_3O_4[b]	251.56 ± 7.59	250.58 ± 1.93	2.90 ± 0.27	30.24 ± 4.11	0.997

Notes: control: no chitosan-Fe_3O_4 added; $P_{measured}$: methane yield; P: maximum methanogenesis potential; R_{max}: maximum methane production rate; λ: methanogenesis lag time; a: methanogenesis kinetics in bath experiment 2; b: methanogenesis kinetics in bath experiment 3. Each sample was analyzed in triplicate.

Multiple acid shocks can inhibit the methanogenesis performance and eventually lead to acidification of the system. Therefore, exploring the restoration effect of CTS-Fe_3O_4 on the anaerobic acid system is necessary. Fig. 2a shows the effects of CTS-Fe_3O_4 and Fe_3O_4 on the methanogenesis performance of the acidified system. The methane yields of CTS-Fe_3O_4, Fe_3O_4, and control reactors were 251.6 mL/gCOD, 109.9 mL/gCOD, and 60.6 mL/gCOD, respectively. These results indicated that the strengthening effects of CTS-Fe_3O_4 were better than Fe_3O_4, the methane yield was increased by 2.3 times, and the methanogenesis lag time was reduced by 26.9%. One reason for this phenomenon may be that excess dissolved iron ions in the acidic environment inhibit the activity of microorganisms. The content of Fe^{2+} in the solution of the Fe_3O_4 reactor was 13.9 mg/L, while that in the CTS-Fe_3O_4 reactor was only 2.9 mg/L (see supplementary materials). Wang et al. (2018) demonstrated that when the concentration of Fe^{2+} in the anaerobic system increased to 10.0 mg/L, the methanogenesis process in the anaerobic system was significantly inhibited. Another reason is that the pH of the CTS-Fe_3O_4 reactor returns to normal more quickly, improving a more suitable environment for methanogenic archaea.

VFA concentrations and pH values are critical environmental factors affecting the stability of anaerobic systems. Therefore, the pH and VFA concentration were determined to understand further the role of CTS-Fe_3O_4 on the AD process under acidic stress. The VFA concentration in the control reactor increased continuously, the total maximum VFA concentration was 1129.1 mg/L, and the system began to undergo further acidification (Fig. 2). For the CTS-Fe_3O_4 group, the total maximum VFA concentration was 786.5 mg/L, and the VFA in the CTS-Fe_3O_4 group was degraded at 120 h. As shown in Fig. 2d, the propionic acid concentration remained high in Fe_3O_4 and control reactors. In the traditional anaerobic digestion process, the oxidation of propionic acid requires hydrogen as an electron carrier to transfer electrons. However, in an acidic anaerobic environment, the interspecies hydrogen transfer pathway between microorganisms is inhibited, leading to a decrease in propionate oxidation efficiency (Zhao et al., 2017b). Therefore, with the progress of AD, the accumulation of propionate and butyrate appeared in the control reactor, which further aggravated the AD performance. Fe_3O_4 can transfer electrons generated during the oxidation of propionate and butyrate, so the concentration of propionate and butyrate in the CTS-Fe_3O_4 reactor is low. These results indicate that CTS-Fe_3O_4 can effectively promote the utilization of fatty acids and improve the system's stability under acidic stress.

To better understand the system's stability, the pH of the reactor was monitored, and the pH of the Fe_3O_4 reactor and the control group was about 5.0. Studies have shown that an anaerobic system pH below 5.3 is not conducive to methanogenesis (Wu et al., 2010; Wu et al., 2020). The low pH of the control group and the Fe_3O_4 reactor inhibited the methanogenesis process, so the system began to acidify further, and the anaerobic digestion performance decreased. However, the pH of the CTS-Fe_3O_4 reactor remained above 6.0, which may be due to the buffering effect of chitosan. Besides, CTS-Fe_3O_4 could also promote the DIET pathway to accelerate the conversion of fatty acids to methane, further improving the system's pH and promoting the restoration of the acidification reactor. Although the pH of the control reactor was adjusted to neutral by alkaline substances, the VFA in the reactor remained at a high level. The pH decreased significantly, suggesting that the acidified system was not recovered by using alkaline chemicals. The characteristics of the sludge were analyzed to reveal the strengthening mechanism of CTS-Fe_3O_4 on the AD of liquor wastewater under acid stress.

3.3. Effects of chitosan-Fe_3O_4 composites on anaerobic sludge under acid stress

3.3.1. Extracellular polymeric substances of anaerobic sludge

Extracellular polymeric substances are the protective layer of microorganisms, which can resist external pressure to microorganisms, and also promote the extracellular electron transfer of microorganisms by secreting electrochemically active substances (Liu et al., 2022; Tang et al., 2021). Therefore, the DOM in EPS was qualitatively analyzed through a three-dimensional excitation-emission matrix (3-DEEM) to understand the mechanism of CTS-Fe_3O_4 improving AD performance. According to the fluorescence characteristics of the compounds and previous studies (Chen et al., 2003), 3-DEEM was divided into five regions. The fluorescence spectrum of EPS in all sludge samples under acid stress is shown in Fig. 3.

Fig. 3 shows the three-dimensional fluorescence of EPS in the control group and the CTS-Fe_3O_4 group. In the CTS-Fe_3O_4 reactor, the peak intensity of microbial by-products and protein-like substances in LB-EPS and the peak intensity of humic substances (HS) in TB-EPS were higher (Fig. 3), indicating that the CTS-Fe_3O_4 can improve the content of microbial by-products and protein-like substances and humic substances. Compared with the reactor without CTS-Fe_3O_4, the TB-EPS protein concentration in the CTS-Fe_3O_4 reactor was 12.7 mg/g VSS, which increased by 18.8%. Torres et al. (2018) found that chitosan could promote anaerobic sludge granulation by increasing the protein content of EPS. According to the study of Su et al. (2020), modifying Fe_3O_4 with amino-rich methionine could increase the biological affinity of Fe_3O_4 and promote the adhesion of microorganisms on the surface of Fe_3O_4 composites. Therefore, the increase of EPS protein in the CTS-Fe_3O_4 reactor may be due to the formation of aggregates. Besides, microorganisms are surrounded by EPS, and EPS can mediate extracellular electron transfer between bacteria and methanogens by facilitating microbial interactions and secreting electroactive substances (Shi et al., 2016). Studies have reported that Fe_3O_4 could enhance the secretion of electroactive substances (humic acid and cytochrome *c*), thus promoting extracellular electron transfer (Liu et al., 2022; Aeschbacher et al., 2010). It can be seen from Fig. 3 that the HS concentration of EPS in the CTS-Fe_3O_4 reactor increased by 10.9%. Moreover, studies have shown that PS includes various cytochrome-c (Cyt-c), and Cyt-c has been shown to mediate electron transport between microorganisms (Liu et al., 2021a). Therefore, the addition of CTS-Fe_3O_4 increased the content of protein and humic acid in EPS. It can be inferred that CTS-Fe_3O_4 can enhance the electron transfer between microorganisms and establish the DIET pathway during AD by promoting the secretion of electroactive substances (Cyt-c and HS) in EPS. Therefore, the reactor with CTS-Fe_3O_4 had a higher VFA conversion rate and methane production rate under acidic stress.

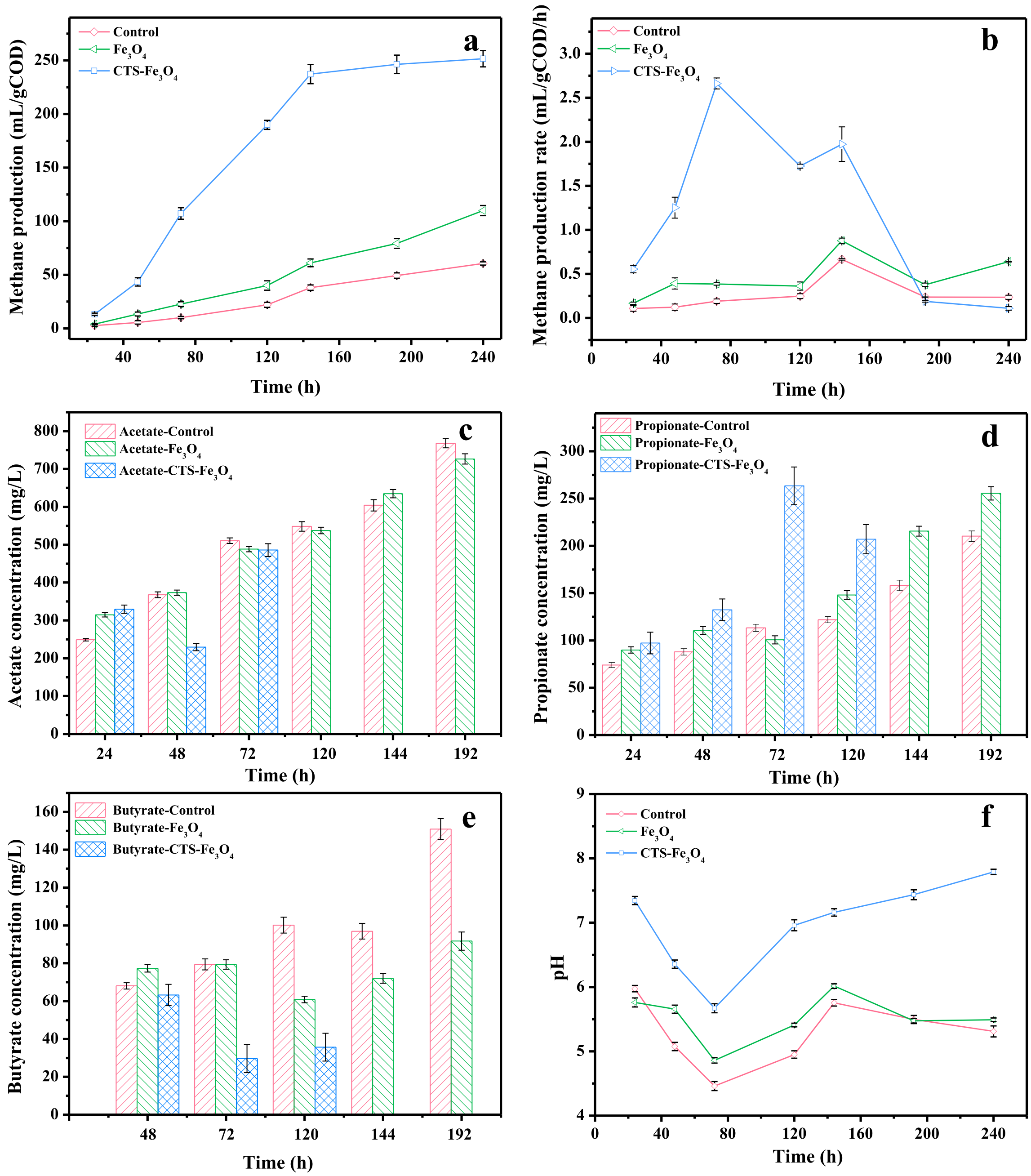

Fig. 2. Effects of chitosan-Fe_3O_4 and Fe_3O_4 addition on acidified anaerobic systems. (a) Methane production; (b) Methane production rate; (c) Acetate; (d) Propionate; (e) Butyrate; (f) pH.

3.3.2. *Electron transfer system activity of anaerobic sludge*

ETS activity can be used to evaluate the respiratory activity of anaerobic microorganisms and is also considered an important indicator to predict the activity of interspecies electron transfer (Liu et al., 2022). Therefore, the ETS activity of microorganisms in the anaerobic system was determined to understand further the role of CTS-Fe_3O_4 on the ETS activity of anaerobic sludge. Fig. 4b shows the effect of CTS-Fe_3O_4 addition on sludge ETS activity under acid stress. CTS-Fe_3O_4 increased ETS activity by 71.4% in the AD of acidic liquor wastewater. Besides, in the acidified anaerobic system, Fe_3O_4 and CTS-Fe_3O_4 increased ETS activity by 64.4% and 94.8%, respectively. The reasons for the enhanced ETS activity in the CTS-Fe_3O_4 reactor are as follows. Firstly, Fe_3O_4 can enhance the electron transfer ability between microorganisms by promoting the secretion of electroactive substances in EPS. Moreover, Fe_3O_4 can replace the conductive pili to directly contact cells and promote electron transfer between bacteria and methanogenic archaea (Wang et al., 2018). At the same time, amino-organic compound modification can further improve the biological affinity of Fe_3O_4 and facilitate the adhesion of microorganisms (Su et al., 2020). Therefore, chitosan modification may also promote the adhesion of microorganisms on the surface of CTS-Fe_3O_4, thus shortening the electron transport distance between microorganisms. Besides, Fe of the CTS-Fe_3O_4 can be utilized by microorganisms to enhance the electron exchange between NADH and NAD^+, enhancing the activity of functional enzymes and ETS (Yang & Wang, 2018). Studies have shown that ETS activity in anaerobic reactors positively correlates with methanogenesis performance (Zhang et al., 2018). These results indicate that adding CTS-Fe_3O_4 can promote syntrophic metabolism by increasing ETS activity under acidic pressure, thereby improving methane production rate and VFA degradation efficiency.

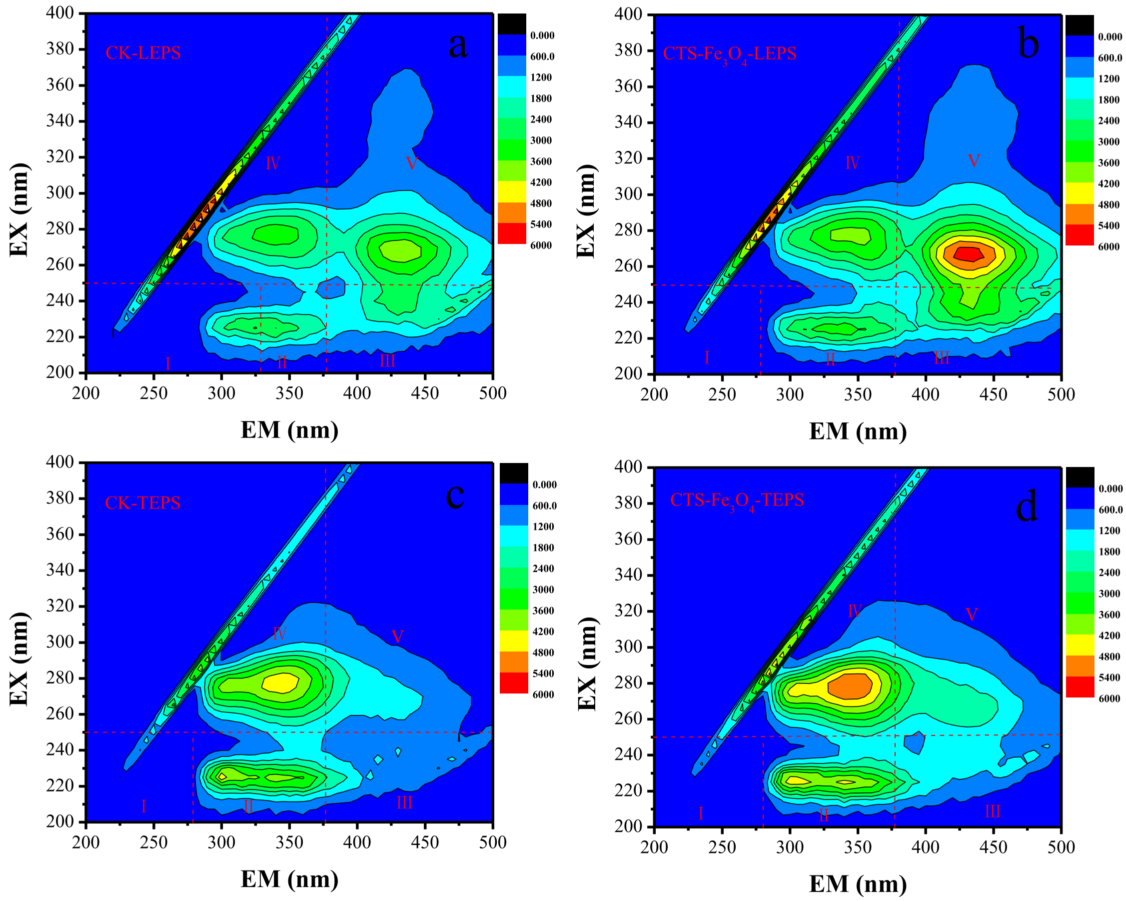

Fig. 3. Excitation emission matrix contours of extracellular polymeric substances from suspended sludge. (a) Loosely bound extracellular polymeric substances from control reactor; (b) Loosely bound extracellular polymeric substances from chitosan-Fe_3O_4 reactor; (c) Tightly bound extracellular polymeric substances from control reactor; (d) Tightly bound extracellular polymeric substances from chitosan-Fe_3O_4 reactor.

3.3.3. *Effects of chitosan-Fe_3O_4 composites on microbial community*

The microbial community structure of reactors was analyzed to understand the effect of CTS-Fe_3O_4 on functional microorganisms under acid stress. Fig. 5a shows the bacterial community composition at the phylum level, and the five main phyla were *Firmicutes*, *Bacteroidota*, *Actinobacteriota*, *Chloroflexi*, and *Patescibacteria*. Compared with the initial bacterial community composition of the sludge (see supplementary materials), the abundance of *Chloroflexi* and *Synergistota* in the CTS-Fe_3O_4 and control reactor decreased after the experiment. *Chloroflexi* has been reported as electroactive microorganisms involved in syntrophy metabolism (Zhang et al., 2018). *Bacteroidota* has been reported to be the dominant bacteria converting glucose and other substances into VFA, H_2, and CO_2 (Zhang et al., 2020b). Besides, *Actinobacteriota* has been reported to degrade various polysaccharides, generating monosaccharides and volatile acids (Ariesyady et al., 2007; Wu et al., 2022). Compared with the control group, the relative abundance of *Actinobacteriota* and *Bacteroidota* increased from 9.5% and 17.2% to 12.9% and 27.6%, respectively, after adding CTS-Fe_3O_4. These results suggested that the CTS-Fe_3O_4 could accelerate the degradation of organic matter by increasing the relative abundance of *Actinobacteriota* and *Bacteroidota* during AD, which is consistent with the enhancement of acidification performance.

The *Peptoclostridium*, *Brooklawnia*, *Bacteroides*, *g_norank_f_Bacteroidetes_vadinHA17* and *Syntrophomonas* were superior bacteria at the genus level (Fig. 5b). The abundance of *Peptoclostridium*, *g_norank_f_Bacteroidetes_vadinHA17*, and *Syntrophomonas* was higher in the CTS-Fe_3O_4 reactor (27.4% vs 22.3%, 19.2% vs 6.6%, 3.2% vs 2.9%). *Peptoclostridium* has key functional genes for syntrophic acetate oxidation (SAO), DIET, conductive pili, and Cyt-c (Westerholm et al., 2016). However, the abundance of *Peptoclostridium* in primary sludge was 3.4%, indicating that the addition of CTS-Fe_3O_4 significantly changed the microbial structure and promoted the enrichment of microorganisms that could participate in DIET. Moreover, SAO by syntrophic acetate-oxidizing bacteria can relieve the inhibition of organic acid on methanogens (Li et al., 2018). Accordingly, CTS-Fe_3O_4 could further enhance SAO to alleviate the inhibition of organic acids on methanogens. The *g_norank_f_Bacteroidetes_vadin HA17* was essential in protein hydrolysis (Liu et al., 2022), and the abundance of *g_norank_f_Bacteroidetes_vadin HA17* in the initial sludge was 12.1%, indicating that adding CTS-Fe_3O_4 promoted the enrichment of hydrolysis bacteria. Besides, *Bacteroidota* and *Syntrophomonas* are electroactive microorganisms involved in DIET, and the accumulation of electroactive microbes is the key to DIET (Liu et al., 2021a). These results reveal that CTS-Fe_3O_4 can increase the abundance of electroactive bacteria, so adding CTS-Fe_3O_4 can construct the DIET pathway between microorganisms DIET to maintain stable methanogenesis under acid stress.

Fig. 5c shows the archaeal community structure in reactors, and the dominant methanogens were *Methanosaeta*, *Methanobacterium,* and *Methanosarcina*. The abundance of *Methanosaeta* in the initial, control, and CTS-Fe_3O_4 reactor was 16.0%, 43.8%, and 52.0%, respectively. *Methanosaeta* is a typical acetoclastic methanogen that can participate in DIET (Liu et al., 2022). The increase in the abundance of *Methanosaeta* indicated that the addition of CTS-Fe_3O_4 promoted acetoclastic methanogenesis. This phenomenon may be because CTS-Fe_3O_4 promoted the process of acetogenesis, resulting in more acetate to acetoclastic methanogen. It is thus plausible that CTS-Fe_3O_4 can reduce the accumulation of acid and increase the pH of the system by promoting acetoclastic methanogenesis. Fig. 5c also shows that the relative proportion

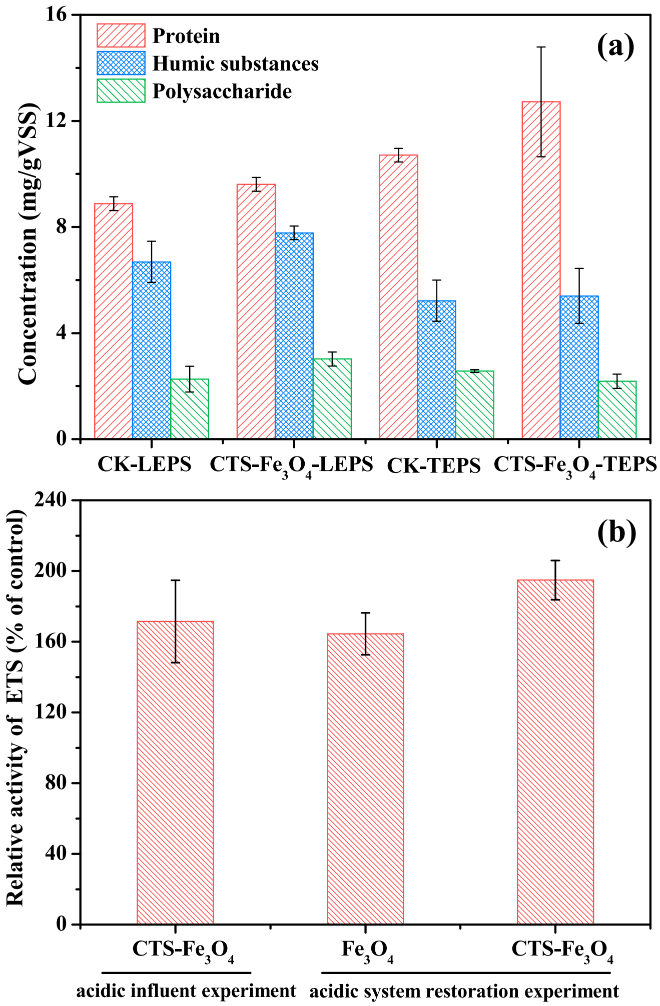

Fig. 4. Effects of chitosan-Fe_3O_4 on sludge properties of anaerobic systems. (a) Protein, polysaccharide, and humic substances content of extracellular polymeric substances; (b) Electron transfer system activity.

of *Methanobacterium* in the CTS-Fe_3O_4 group dropped from 37.4% to 33.8%. Methanobacterium, as a hydrogenotrophic methanogen, maintains the hydrogen pressure of the system by producing methane from H_2 (Wang et al., 2020). Besides, *Methanobacterium* is also a typical methanogen for interspecies electron transfer via H_2 (Zheng et al., 2020). Therefore, the decrease in *Methanobacterium* abundance indicated that adding CTS-Fe_3O_4 changed the interspecies electron transfer pathway from IHT to DIET in the anaerobic acid system, which is similar to the findings of Zhao et al. (2017b). These findings indicate that CTS-Fe_3O_4 can also mitigate acid inhibition by promoting the shift of the traditional IHT pathway to the DIET pathway.

To further understand the effect of CTS-Fe_3O_4 on microorganisms during anaerobic digestion processes, a magnet was used to recover CTS-Fe_3O_4 from the reactor, and the microbial community on the surface of CTS-Fe_3O_4 was analyzed (see supplementary materials). For the bacteria community, the relative abundance of bacteria varied considerably between the CTS-Fe_3O_4 surface and the CTS-Fe_3O_4 reactor. *Firmicutes* (32.5%) and *Proteobacteria* (22.2%) were the main bacterial communities on the surface of CTS-Fe_3O_4. *Proteobacteria* is the dominant bacteria in digesting glucose and fatty acids (Ariesyady et al., 2007), and *Proteobacteria* also contains electroactive *Geobacter* (Lovley et al., 2004). For archaeal communities, the abundance of *Methanobacterium* was 2.0%. These results indicated that CTS-Fe_3O_4 could effectively retain the microorganisms capable of hydrolysis, acidification, and methanogenesis. In addition, for electroactive microorganisms closely adsorbed on CTS-Fe_3O_4, DIET is more likely to occur between these microorganisms (Su et al., 2020). These results suggest that CTS-Fe_3O_4 can also accelerate electron transfer between microorganisms by attaching electroactive microorganisms and methanogens.

3.4. Possible mechanisms and application potential

The proposed mechanism of CTS-Fe_3O_4 improving AD performance under acidic pressure is as follows. First, CTS is rich in amino groups, which can be combined with hydrogen ions to buffer acid inhibition. In addition, the combination of amino groups and hydrogen ions can positively charge the surface of CTS (Torres et al., 2018), thereby enhancing the aggregation of microorganisms on the surface of CTS-Fe_3O_4. Secondly, Fe_3O_4 in CTS-Fe_3O_4 can promote the extracellular electron transfer between microorganisms by promoting the secretion of electroactive substances in EPS. Besides, the microorganisms attached to CTS-Fe_3O_4 are more conducive to promoting syntrophic metabolism. The change in VFA concentration indicated that CTS-Fe_3O_4 promoted the oxidation of propionic acid and butyric acid under acidic pressure, accelerated the degradation of fatty acids, and promoted the restoration of the system. Finally, CTS-Fe_3O_4 increased the abundance of electroactive microorganisms, especially syntrophic acetate-oxidizing bacteria and acetoclastic methanogens. These findings suggested that CTS-Fe_3O_4 could selectively enrich functional microorganisms under acidic pressure to alleviate acidic pressure.

In other studies, Wu et al. (2020) found that adding graphene increased methane production by 11.0% under acid shock, while pyrochar did not increase methane production. This work improved the AD performance of liquor wastewater under acidic pressure by adding CTS-Fe_3O_4, and the methane production increased by 2.3–4.2 times. Besides, Domrongpokkaphan et al. (2021) found that adding 1.6 g/g VSS zerovalent iron could improve the AD performance of acid wastewater, but the dissolved iron concentration reached about 130.0 mg/L. In this study, CTS-Fe_3O_4 had good stability and low iron ion dissolution concentration in the CTS-Fe_3O_4 reactor. Although Fe_3O_4 can also promote methane production, the dissolution of many iron ions in the acidic environment may further inhibit microbial activity, resulting in low biomethane production. It is worth noting that the amino group in CTS can bind to hydrogen ions in an acidic environment, thereby promoting the formation of sludge aggregates (Torres et al., 2018). Therefore, adding CTS-Fe_3O_4 to the continuous anaerobic reactor may also promote sludge granulation and improve the stability of the reactor. Moreover, in the sludge purging process, CTS-Fe_3O_4 in sludge can be recycled and reused by magnetic recovery to reduce operating costs. Therefore, it is feasible to alleviate acid inhibition and restore the anaerobic acidification system by adding CTS-Fe_3O_4.

4. Conclusions

This research showed that adding CTS-Fe_3O_4 could improve the AD performance of liquor wastewater. Adding 0.5 g/g VSS CTS-Fe_3O_4 increased the methane yield by 1.5–2.3 times and accelerated the restoration of the acidic anaerobic system. Firstly, CTS could buffer acid inhibition and promote microbial enrichment on the surface of the composite material. Besides, Fe_3O_4 could increase the electron transfer between anaerobic microorganisms and accelerate fatty acid conversion. Therefore, the AD performance improvement by CTS-Fe_3O_4 was attributed to the synergistic effect of Fe_3O_4 and CTS. These results may offer valuable insights for improving AD performance under acidic stress.

CRediT authorship contribution statement

Wenkai Nie: Conceptualization, Methodology, Data curation, Visualization, Formal analysis, Writing – original draft, Writing – review & editing. **Yan Lin:** Investigation, Formal analysis, Writing – original draft.

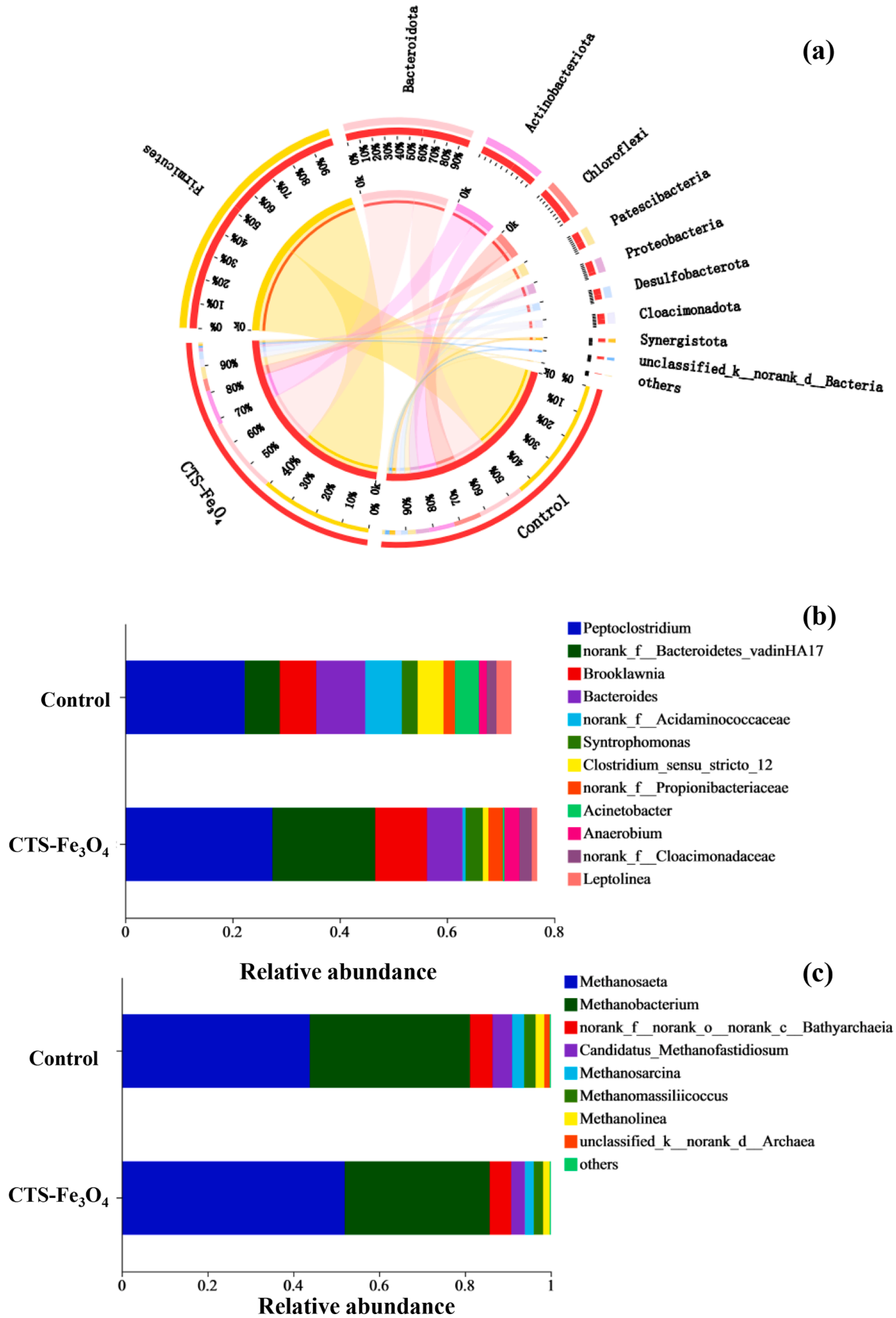

Fig. 5. Composition of microbial community in sludge samples under acidic pressure. (a) Bacteria at phylum level; (b) Bacteria at genus level; (c) Archaea at genus level.

Xin Wu: Methodology, Formal analysis, Writing – original draft. **Shaohua Wu:** Methodology, Formal analysis, Writing – original draft. **Xiang Li:** Methodology, Formal analysis, Writing – original draft. **Jay J. Cheng:** Methodology, Formal analysis, Writing – original draft, Writing – review & editing. **Chunping Yang:** Conceptualization, Methodology, Funding acquisition, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2023.128927.

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