

Article

Methane Production from Anaerobic Digestion of Sludge Enhanced by Calcium Hypochlorite Treatment with Zero-Valent Iron Regulation

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Abstract

Calcium hypochlorite (CH) is a strong oxidant that can be utilized to enhance sludge hydrolysis and anaerobic digestion (AD) performance; however, its suppressive effect on methanogens restricts the development of CH treatment technologies. This laboratory-scale study introduced zero-valent iron (ZVI) into a CH-treated sludge AD system to alleviate the adverse effect, thereby synergistically enhancing biomethane production, and the correlation between methane yield and the two key treatment parameters (ZVI and CH dosages) was explored. The experimental results revealed that the best conditions for the ZVI + CH method were 5 g/L of ZVI plus 0.12 g/g of volatile suspended solids (VSSs) of CH, under which the maximum biomethane yield of 275.8 mL/g VSS was achieved, representing increases of 81.5%, 43.2%, and 28.2% over the control, solo ZVI, and solo CH conditions, respectively. More organic matter in the sludge was found to be degraded during AD by the ZVI + CH treatment compared with the control, solo ZVI, or solo CH conditions. An enzyme activity analysis illustrated that the ZVI + CH treatment not only enhanced the bioactivity of anaerobes but also eliminated the suppression of methanogens by CH. A microbial analysis demonstrated that all functional microbes responsible for sludge AD were enriched by the ZVI + CH treatment, with total abundances of 8.41% and 20.58% in the control and ZVI + CH-treated reactors, respectively.

Keywords: organic solid waste; anaerobic digestion; regulation; biogas



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1. Introduction

Nowadays, the proper disposal of the ever-increasing waste activated sludge (WAS) generated by sewage-treatment plants (STPs) has become an urgent issue. As a promising sludge-treatment technology, anaerobic digestion (AD) enables both the decomposition of organic matter and the generation of useful byproducts, e.g., methane [1], volatile fatty acids (VFAs) [2] and hydrogen [3]. In other words, the stabilization and resource recovery of sludge are synchronously accomplished via AD. However, the traditional AD process of sludge is generally less efficient, which restricts its large-scale application.

The main reason for the low efficiency of sludge AD is the slow reaction rate of the hydrolysis process. To improve this drawback, many pretreatment technologies have been proposed to accelerate such rate-limiting steps, largely enhancing sludge AD performance in recent years, and these are generally grouped into three types, i.e., chemical, physical, and biological methods [4]. Among them, chemical oxidization methods have drawn widespread attention because of their merits of high efficiency, operational simplicity and low capital requirements [5,6]. Various chemical oxidants, such as persulfate [7], potassium ferrate [8], ozone [9], and calcium peroxide [10], were found to be valid in enhancing sludge AD. On the one hand, the oxidants can directly destroy the extracellular polymeric substances (EPSs) of sludge and break microbial cells, causing the releasement of organic matter from the solid to the liquid phase. On the other hand, dissolved organic matter with high molecular weight can be degraded into simple compounds in the oxidization process, which are more available for utilization by fermentative microbes [11].

Calcium hypochlorite (CH) is a strong oxidant that is widely applied in water treatments [12,13] and which can effectively degrade different kinds of pollutants and kill pathogenic bacteria. The potential of CH to be applied in sludge treatment, especially for pretreatment before sludge AD to enhance hydrolysis efficiency, has been explored in recent years [14,15]. For instance, the soluble chemical oxygen demand (SCOD) concentration of sludge was observed to largely increase from 0.1 to 0.34 g/g volatile solids (VSs) after 0.24 g/g VS of CH treatment [14], realizing an increase rate of 59.7% for methane production in the subsequent AD process. It was reported by Tang et al. [15] that the EPSs of sludge and microbial cells were both effectively destroyed by a 75 mg/g volatile suspended solids (VSSs) CH treatment, accompanied by a marked increase in soluble organic matter content. Consequently, the methane yield from sludge AD was enhanced substantially from 169.3 to 231.8 mL/g VSS with CH treatment. Though the excellent performance of CH in improving biomethane production from sludge has been extensively proved, several recently reported studies showed that the excessive addition of CH can result in suppressive effects on sludge AD [16,17]. For example, it was found that the methane yield of sludge AD initially increased when CH content was raised from 0 to 0.1 g/g total suspended solids (TSSs) and then decreased with further increased CH dosages [16]. The initial rise and subsequent decline trend of biomethane yield with enhanced CH dosage was also reported in our previous work [17], and the main reason is that different kinds of methanogens (e.g., *Methanolinea* and *Methanosaeta*) were generally suppressed by CH, especially at high CH dosages. Therefore, an additional method is needed to help CH alleviate its adverse effect on methanogenesis and further enhance sludge AD performance.

Zero-valent iron (ZVI) is a commonly used, low-cost reductive material that can be easily obtained in daily life, and its application in sludge AD for enhancing methane production has attracted a lot of attention in recent years [18,19]. For example, the methane yield in sludge AD was found to be enhanced by between 9.6% and 43.5% with the addition of ZVI at dosages of 1 to 20 g/L [18]. In another study [19], the best ZVI dosage for the AD of primary sludge was found to be 4 g/L, at which the biomethane yield was enhanced by 26.9%. The main mechanisms by which ZVI reinforced sludge AD are as follows: (1) ZVI can reduce the oxidative–reductive potential (ORP) of an AD system [20], which is conducive to the growth of anaerobic bacteria. (2) ZVI, with excellent electrical conductivity, establishes a new direct interspecies electron transfer (DIET) pathway, thereby improving electron transfer efficiency among functional microbes and strengthening the degradation of organic matter [21]. (3) ZVI undergoes hydrogen evolution corrosion to produce exogenous hydrogen, which can not only enhance energy and electron transfer among microorganisms but also stimulate the enrichment of hydrogenotrophic methanogens [18]. Moreover, the mitigative effect of ZVI on some adverse factors of sludge AD, such as

VFAs accumulation [22], pH variation [23], and high contents of ammonia, sulfate and phosphate [24–26], has also been reported recently. For instance, ZVI was integrated with calcium peroxide to reinforce sludge AD [27], and the results showed that the methane yield under the co-treatment condition was 9.7% and 13.8% higher than those of solo calcium peroxide and solo ZVI methods, respectively. Therefore, it was hypothesized that the AD performance of sludge could be further enhanced by using ZVI to assist CH treatment, because the adverse effect of CH on methanogenesis would be relieved, and higher metabolic efficiency would be realized. Nevertheless, this issue has remained unexplored to date.

This study aims to reveal whether ZVI can assist CH to synergistically enhance the methane yield of sludge AD, and to explore the underlying mechanisms. Firstly, methane production from sludge treated by the combined method, with different dosages of ZVI (1, 5, and 10 g/L) and CH (0.03 to 0.15 g/g VSS), was investigated. Then, the underlying mechanism by which ZVI + CH enhance sludge AD performance was explored in terms of organic matter degradation, VFAs consumption, key enzyme activities and microbial communities. Finally, a technical analysis of the ZVI + CH method was performed by comparing its methane increase rate with those of other CH-based methods reported in the previous literature.

2. Materials and Methods

2.1. Sludge and Chemicals

Raw sludge was sampled from a sewage-treatment plant (STP) in Jingzhou, China. The inoculum was obtained from a laboratory-scale anaerobic reactor in continuous operation, with sludge being the only fermentation substrate. Table 1 shows the main characteristics of the raw sludge and inoculum. CH (60–75% free chlorine) and ZVI (99%, 100 mesh) reagents were acquired from Adamas Company in Shanghai, China.

Table 1. The main characteristics of raw sludge, inoculum, and their mixtures used in this study.

Parameters	WAS	Inoculum	Mixture of WAS and Inoculum (1:1)
pH	6.8 ± 0.1	7.3 ± 0.1	7.1 ± 0.1
TSSs (total suspended solids) (mg/L)	25,744 ± 148	21,341 ± 123	23,480 ± 155
VSSs (volatile suspended solids) (mg/L)	16,476 ± 130	10,279 ± 106	13,304 ± 128
TCOD (total chemical oxygen demand) (mg/L)	25,062 ± 215	15,735 ± 193	20,217 ± 220
SCOD (soluble chemical oxygen demand) (mg/L)	130 ± 6	367 ± 10	261 ± 9
Total protein (mg/L)	5465 ± 38	–	–
Total polysaccharide (mg/L)	2270 ± 33	–	–

2.2. Revealing the Optimal Conditions for ZVI-Assisted CH Treatment

This experiment was conducted to determine the optimal dosages of ZVI and CH for methane production from sludge AD, with ZVI tested at 1, 5, and 10 g/L and CH at 0.03, 0.06, 0.09, 0.12, and 0.15 g/g VSS. The three ZVI dosages were adopted based on the previous literature [27–29]. The CH dosage range (0.03 to 0.15 g/g VSS) was determined according to the results of a preliminary experiment (Figure 1), as the methane yield first enhanced and then decreased with increased CH dosage in this range. To cover all fifteen combinations of ZVI and CH dosages, sixteen serum bottles (V = 1 L) were used as anaerobic reactors: one served as the control group (Reactor 1) and fifteen were treated with different ZVI/CH combinations (Reactors 2–16). Firstly, each reactor received 300 mL of raw sludge. Then, the fifteen experimental reactors, excluding Reactor 1, received different dosages of CH, as listed in Table 2. After the CH and sludge were thoroughly mixed with an impeller agitator (600 rpm) for 24 h, ZVI was introduced to the reactors at different

concentrations based on Table 2. Subsequently, each reactor received 300 mL of inoculum to reach a substrate–inoculum ratio of 1:1 (volume ratio) and was purged with nitrogen gas (N₂) to eliminate the internal air. Finally, the reactors were sealed and placed in a shaking incubator (150 rpm, 35 °C) for AD reaction. The methane yield from each reactor was detected daily during AD based on the method reported in our previous study [30], and the optimal dosages of ZVI and CH were determined by comparing the cumulative methane production among different reactors.

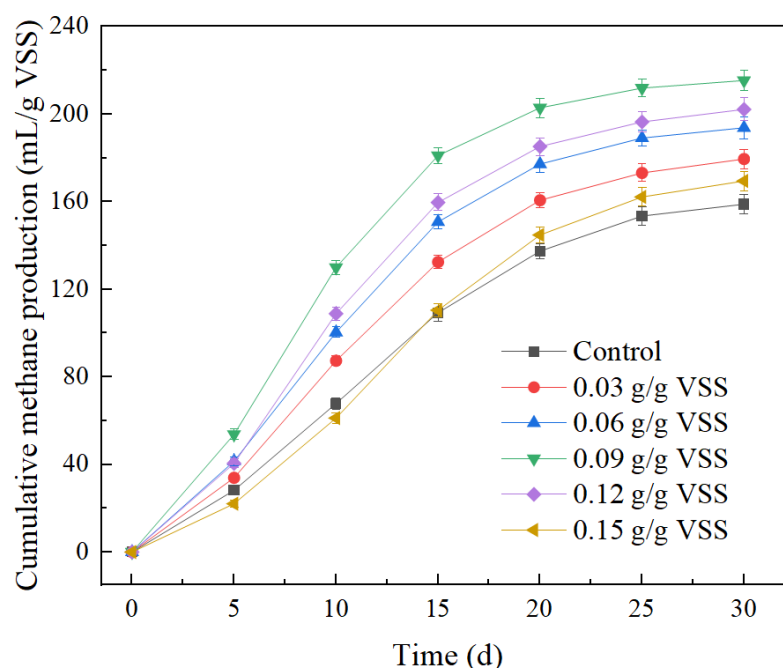


Figure 1. Cumulative methane production from sludge with the addition of different CH dosages. Error bars represent standard deviations of triplicate tests.

Table 2. ZVI and CH dosages in Reactors 1–16.

Reactors	CH Dosage (g/g VSS)	ZVI Dosage (g/L)
1	0	0
2	0.03	1
3	0.06	1
4	0.09	1
5	0.12	1
6	0.15	1
7	0.03	5
8	0.06	5
9	0.09	5
10	0.12	5
11	0.15	5
12	0.03	10
13	0.06	10
14	0.09	10
15	0.12	10
16	0.15	10

2.3. Revealing Whether ZVI-Assisted CH Treatment Had a Synergistic Effect on Sludge AD

This experiment aimed to verify whether the ZVI + CH method could synergistically enhance methane yield from sludge AD and to expose the underlying mechanisms. The optimal combination (5 g/L ZVI + 0.12 g/g VSS CH) determined in Section 2.2 was used to explore the synergistic effect herein. Four serum bottles were adopted as AD reactors, named Reactor 17 to 20. Each reactor first received 300 mL of raw sludge. Following the steps in Section 2.2, Reactor 17 was left untreated as the control, while Reactor 18, Reactor 19 and Reactor 20 were treated with solo ZVI (5 g/L), solo CH (0.12 g/g VSS) and ZVI + CH (5 g/L of ZVI + 0.12 g/g VSS of CH), respectively. Finally, each reactor received 300 mL of inoculum, and then the AD reaction was initiated according to Section 2.2. The methane production and the concentrations of key AD parameters (e.g., VFAs, COD, polysaccharide, and protein) were periodically detected during the AD process. Based on the practical methane yield data, a modified Gompertz model was used to simulate the key kinetic parameters of sludge AD, and the specific method was the same as that reported in our previous work [30].

2.4. Analytical Methods

COD, VSS and TSS were detected using standard methods [31]. Protein and polysaccharide were quantified following the analytical procedures described in the literature [32,33]. VFA concentrations and biogas components (i.e., methane, hydrogen and carbon dioxide) were detected using a gas chromatograph (Shimadzu GC-2010, Kyoto, Japan), and the parameters and specific methods were the same as those in our previous study [30]. The INT (2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride) method was employed to determine the electron transport system (ETS), as reported in the literature [34]. Coenzyme F₄₂₀ activity was detected according to the previously reported method [35]. The microbial communities were analyzed using the Illumina Miseq sequencing technique, and the specific procedures were consistent with those in our previous study [30,36,37]. Using a 97% sequence similarity threshold, the raw reads were grouped into operational taxonomic units (OTUs). All experiments in this study were conducted in triplicate, and the results are expressed as mean $\pm$ standard deviation in the figures and tables. The significance of the results was tested by variance analysis.

3. Results and Discussion

3.1. Confirmation of the Optimal Conditions for ZVI-Assisted CH Treatment

Figure 2 shows the cumulative methane production from the sludge AD reactors with different ZVI and CH concentrations. Compared with the control (without the addition of ZVI or CH), all ZVI + CH combinations resulted in an obvious enhancement in methane yield, and the yield was influenced by the dosages of both additives. At a constant ZVI level of 1 g/L, the methane yield exhibited an initial rise and subsequent declining trend when the CH content increased from 0.03 to 0.15 g/g VSS. Similar trends occurred at ZVI dosages of 5 and 10 g/L. Meanwhile, the methane yield varied considerably with changing ZVI dosage. At a CH content of 0.03 g/g VSS, the methane yield gradually increased with enhanced ZVI dosage from 1 to 10 g/L. In contrast, an initial increase followed by a decreasing trend in methane production with enhanced ZVI dosage was observed when CH dosage was constant at 0.12 g/g VSS. By comparing the methane yield under different conditions, the optimal condition of ZVI + CH treatment was 5 g/L of ZVI plus 0.12 g/g VSS of CH, which achieved the highest methane yield of 277.1 mL/g VSS ($p < 0.05$). Therefore, this combination (5 g/L ZVI + 0.12 g/g VSS CH) was utilized for further analysis in the following context.

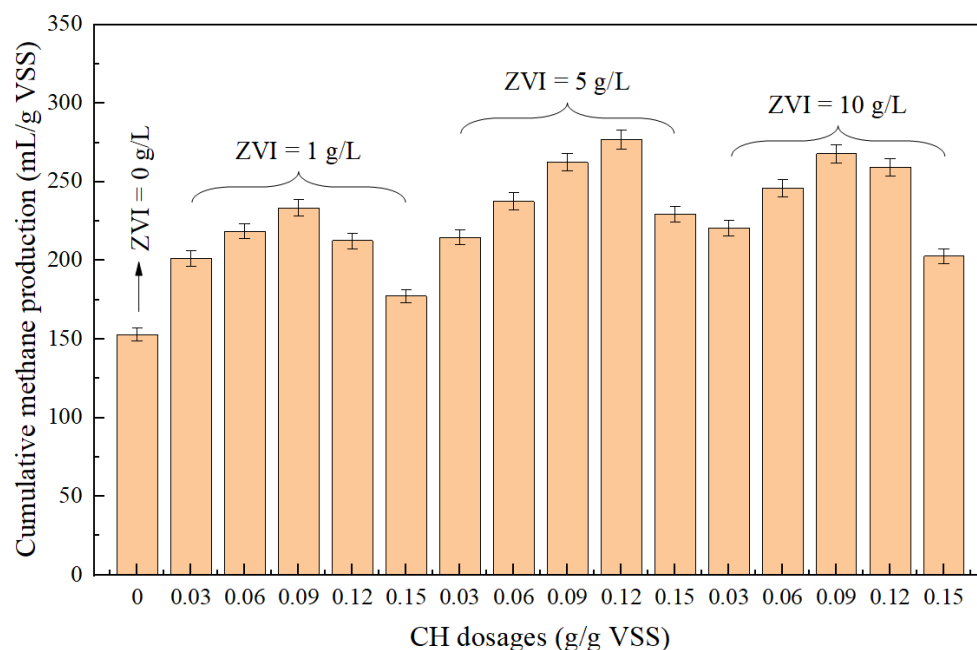


Figure 2. Cumulative methane production from AD of sludge treated with different ZVI/CH combinations. Error bars represent standard deviations of triplicate tests.

3.2. The Synergistic Effect of ZVI-Assisted CH Treatment on Sludge AD Performance

The levels of cumulative methane production over time from sludge AD under the control, solo ZVI, solo CH and ZVI + CH conditions are depicted in Figure 3a. No marked rise in cumulative methane yield was observed under these conditions after 33 days, which means that the AD reactions of different sludge samples were completed. A relatively low level of methane production was detected in the control (152.0 mL/g VSS), which was similar to those reported in relevant studies and indicated that the AD of sludge was inadequate without treatment [38,39]. When treated with solo ZVI or solo CH, methane production increased to 192.6 and 215.2 mL/g VSS, respectively, which are significantly higher than that of the control group (p values were 0.009 and 0.004, respectively). Under the ZVI + CH condition, methane yield further increased to 275.8 mL/g VSS, which was 81.5%, 43.2%, and 28.2% higher than the values for the control, solo ZVI, and solo CH, respectively. The p values for methane yield were 0.001 (ZVI + CH versus solo ZVI) and 0.003 (ZVI + CH versus solo CH), respectively, indicating significant enhancement. The analysis above indicated that both ZVI and CH methods were sufficient for enhancing sludge AD performance, and their combination achieved a synergistic effect. As reported in the literature [17], CH can not only accelerate sludge hydrolysis but also promote the biodegradability of the organic matter in sludge, generating abundant fermentation substrates for methane production. For ZVI, its excellent performance in enhancing microbial growth and metabolism has been widely reported [18,20,21], and could be the primary reason for the increased methane production by ZVI treatment in this study. Therefore, the mechanism of the synergistic increase in methane yield by ZVI + CH treatment was investigated from the perspective of both organic matter utilization and microbial growth and metabolism in the following context.

The modified Gompertz model was then used to simulate methane production data, thereby revealing the mechanism by which ZVI + CH synergistically enhances sludge AD performance in terms of reaction kinetics. Figure 3b depicts the fitted curves of the model in different situations, and the specific fitting results are presented in Table 3. From Figure 3b, the simulative curves were very close to the actual values, and the relevant R^2 values were generally higher than 0.99 (Table 3), indicating that the model reflected the AD reactions

of sludge under different conditions well. Also from Table 3, the three kinetic parameters (i.e., methane production potential (M_m), methane production rate (R_m), and lag phase time (λ)) all varied considerably under different treatment conditions. The M_m increased from 152.4 mL/g VSS in the control to 197.4 and 219.1 mL/g VSS under the solo ZVI and solo CH treatments, respectively. When treated with ZVI + CH, the M_m further increased to 278.3 mL/g VSS, suggesting that the methane production potential was synergistically enhanced by the combined treatment. A comparable tendency was noted for R_m . The value increased from 10.9 to 12.9 and 14.7 mL/g VSS/d with solo ZVI and solo CH treatments, respectively, and reached 22.4 mL/g VSS/d by ZVI + CH, indicating that the combined treatment also synergistically increased the methane production rate.

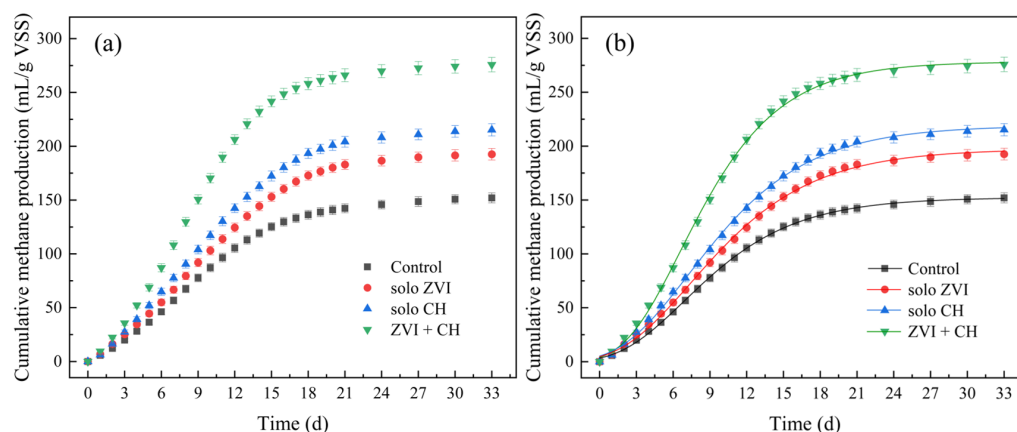


Figure 3. Cumulative methane productions from the control, solo ZVI, solo CH, and ZVI + CH reactors (a) and corresponding fitted curves of the modified Gompertz model (b). Error bars represent standard deviations of triplicate tests.

Table 3. Kinetic parameters of the modified Gompertz model under different treatment conditions.

Treatment Conditions	Kinetic Model Parameters			
	M_m (mL/g VSS)	R_m (mL/g VSS/d)	λ (d)	R^2
Control	152.4 ± 0.6	10.9 ± 0.1	1.8 ± 0.1	0.9995
Solo ZVI	197.4 ± 1.3	12.9 ± 0.2	1.7 ± 0.1	0.9987
Solo CH	219.1 ± 1.4	14.7 ± 0.2	2.0 ± 0.2	0.9989
ZVI + CH	278.3 ± 1.4	22.4 ± 0.4	1.7 ± 0.1	0.9990

M_m —methane production potential; R_m —methane production rate; λ —lag phase time.

3.3. The Effect of ZVI-Assisted CH Treatment on the Utilization of Organic Matter

Higher methane production means that more organic matter might be degraded under the ZVI + CH condition. Therefore, the concentrations of total chemical oxygen demand (TCOD), protein and polysaccharide in different reactors after AD were detected, and the corresponding degradation rates were calculated, as presented in Figure 4. The degradation rate of TCOD was only 35.2% in the control group, which also demonstrates the insufficient AD reaction of sludge in this reactor. When treated with solo ZVI or solo CH, this value increased to 41.1% and 46.5%, respectively, and the highest value of 54.7% was obtained under the ZVI + CH condition (Figure 4a). Protein and polysaccharide are the primary types of biodegradable organic matter contained in sludge, and their degradation rates showed patterns similar to that of TCOD (Figure 4b,c). For example, the degradation rate of protein first enhanced from 38.4% to 43.6% and 51.4% by solo ZVI and solo CH treatments, respectively, and further increased to 59.6% under the ZVI + CH condition (Figure 4b). The results above illustrate that both ZVI and CH reinforced the degradation of organic matter in sludge, especially the biodegradable fraction, and their combination achieved synergistic

effects. According to the literature [40], ZVI can facilitate the growth of anaerobic bacteria by creating a low-ORP environment. In this study, the ORPs in the control, solo ZVI, solo CH and ZVI + CH reactors after treatment were measured to be -124 , -227 , 105 , and -118 mV, respectively. The results suggested that the presence of ZVI not only reduced the ORP in the solo treatment but also alleviated the adverse effects of residual oxidizing substances from CH in the co-treatment, resulting in the enhanced degradation of AD substrates (Figure 4). Our previous work showed that CH can destroy the EPS structure of sludge and damage microbial cells [41], resulting in the migration of organic matter from the solid phase into the liquid phase. Figure 5a shows the SCOD contents in different reactors after treatment. Compared with the control, the SCOD contents largely increased with the solo CH and ZVI + CH treatments, suggesting that abundant dissolved organic matter was generated under these conditions. The dissolved substances are more easily utilized by the functional microbes responsible for AD, which accounts for the main reason for the enhanced organic matter degradation by the CH treatment in this study. According to the above analysis, the mechanism by which ZVI + CH synergistically promoted the degradation of organic matter in sludge was revealed.

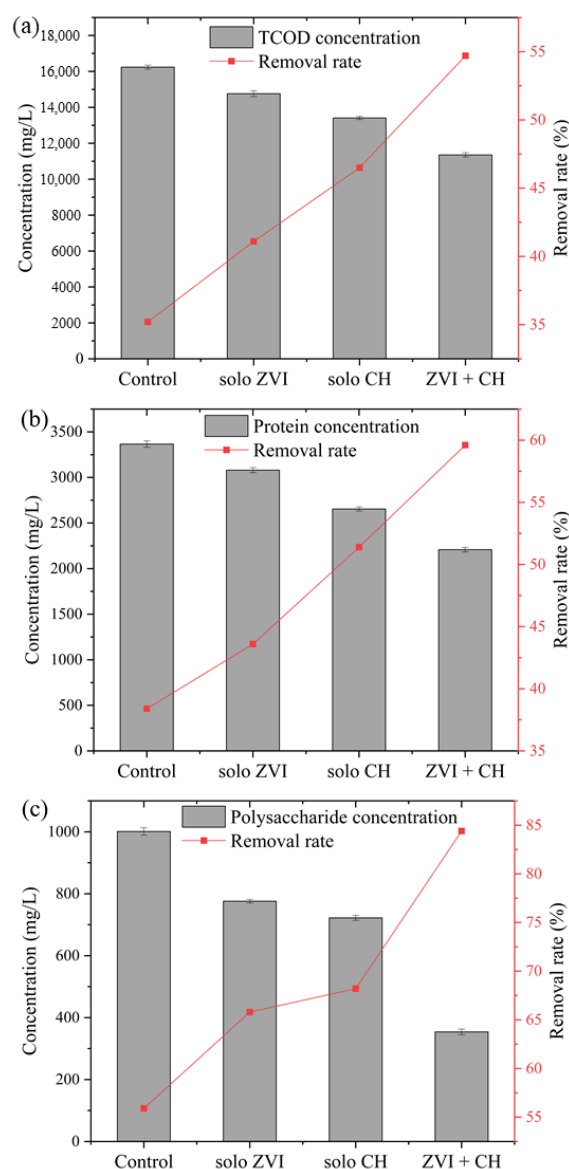


Figure 4. Concentrations and removal rates of TCOD (a), protein (b), and polysaccharide (c) in different reactors when AD was completed. Error bars represent standard deviations of triplicate tests.

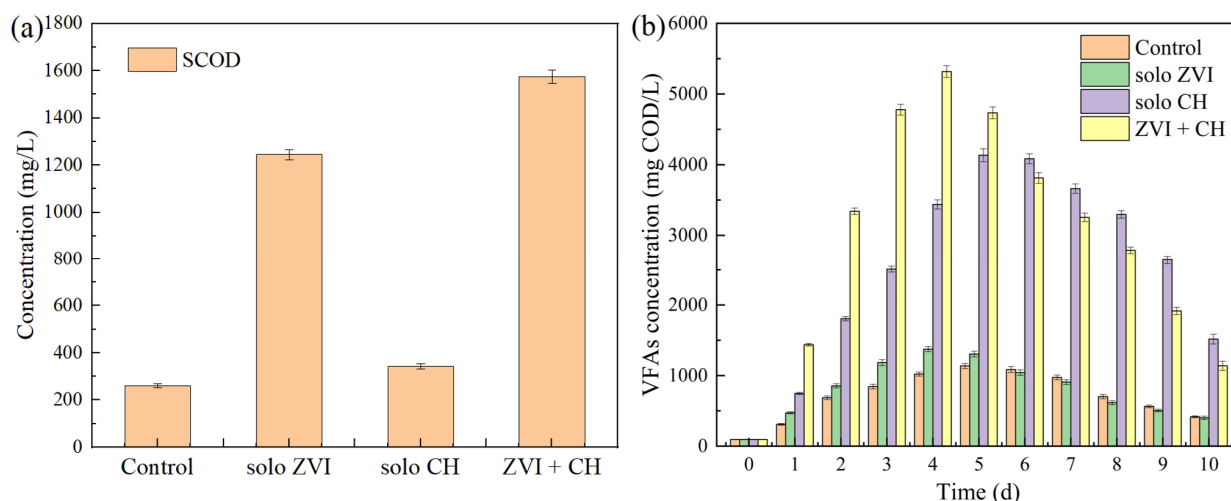


Figure 5. The SCOD contents of sludge samples with or without treatment (a) and the variations in VFA concentrations over time during the initial 10 days of AD in different reactors (b). Error bars represent standard deviations of triplicate tests.

As important intermediate products in sludge AD systems, VFAs are generated from the acidification of organic substances in sludge and can be further utilized for methane generation via methanogenesis. Figure 5b shows the variations in VFA concentrations over time during the initial period of AD in different reactors. A trend of rapid increase followed by a decrease was observed in each reactor, and the maximum VFA concentrations in different reactors were generally reached on the fourth or fifth day. Among all the reactors, the maximum VFA concentration of 5317.5 mg/L was detected in the ZVI + CH-treated reactor on the fourth day, which was significantly higher than the values of 1136.2, 1375.1 and 4134.8 mg/L in the control, solo ZVI and solo CH reactors, respectively. It was also observed that, after 5 days of AD, the VFA concentrations in the solo ZVI and ZVI + CH reactors were lower than those in the corresponding reactors without ZVI addition (i.e., control and solo CH, respectively), which means that the presence of ZVI facilitated the utilization of VFAs. This phenomenon was also observed in the previous study [18], as ZVI accelerated the conversion of VFAs to methane and thus avoided the excessive accumulation of VFAs. Based on the analysis above, the ZVI + CH method not only enhanced sludge hydrolysis efficiency, thereby generating abundant fermentation substrates and intermediate products, but also reinforced the utilization of these substances, resulting in a significant increase in methane production.

3.4. The Effect of ZVI-Assisted CH Treatment on the Activity of Microbes

Sludge AD reactions involve complex electron transfer processes; thus, the ETS's activity is widely adopted to evaluate the bioactivity of anaerobes [42–44]. Figure 6 shows the relative ETS activities in the reactors with or without treatment. Compared with the control group, the reactors treated with solo ZVI, solo CH, or ZVI + CH all exhibited significant increases. For example, the ETS activity in the solo CH-treated reactor was 18.4% above the control level. The possible reason is that CH treatment largely enhances sludge hydrolysis efficiency, providing more fermentation substrates for anaerobes, and thereby enhancing their bioactivity. It should be noted that the solo ZVI-treated reactor achieved higher ETS activity than the solo CH reactor, and the highest value was obtained in the ZVI + CH-treated reactor, which means that the combination of ZVI and CH synergistically enhanced the bioactivity of the anaerobes. As reported in the literature [21], ZVI can largely enhance electron transfer efficiency through DIET in a sludge AD system, which serves as the possible reason for the enhanced ETS activities observed under the solo

ZVI and ZVI + CH conditions. The activity of coenzyme F_{420} , a key indicator reflecting the activity of methanogens, was also detected in the reactors with or without treatment (Figure 6). Distinct results were observed under different conditions, as the solo ZVI treatment enhanced the activity of coenzyme F_{420} while the solo CH treatment reduced it. This indicates that the activity of methanogens was improved by the solo ZVI treatment, whereas a suppressive effect was caused by the solo CH treatment. In our previous work [45], the ORP and pH of the sludge AD system were both varied by CH treatment, which could be a possible reason for the suppression of methanogen activity by CH, as these archaea are highly susceptible to environmental fluctuations. On the contrary, ZVI can not only decrease the ORP of an AD system but can also produce hydrogen through hydrogen evolution corrosion [21], both of which are beneficial to the growth of methanogens. Also, as shown in Figure 6, the activity of coenzyme F_{420} in the ZVI + CH-treated reactor was close to that in the solo ZVI reactor, indicating that the adverse effect of CH on methanogens was relieved with the assistance of ZVI.

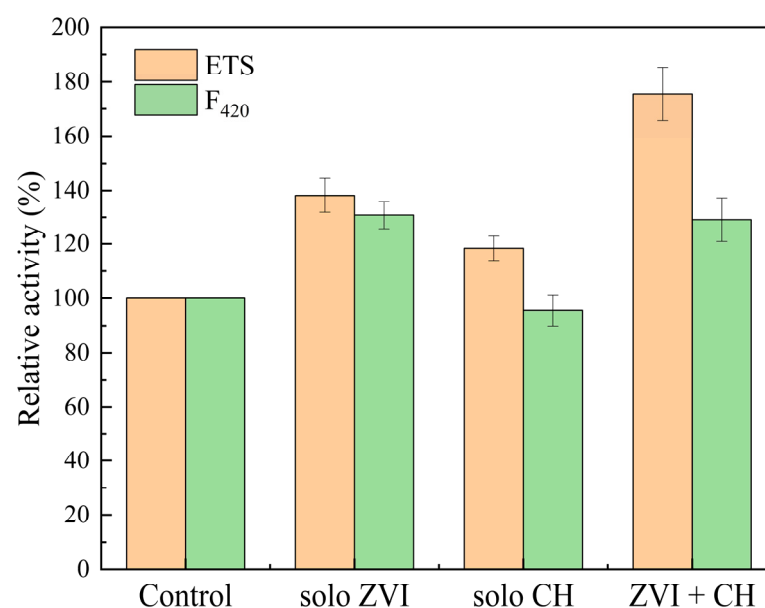


Figure 6. The relative activities of ETS and coenzyme F_{420} in the solo ZVI-, solo CH-, and ZVI + CH-treated reactors compared with the control.

3.5. The Effect of ZVI-Assisted CH Treatment on Microbial Communities

To reveal the effect of the ZVI + CH treatment on specific functional microbes that participate in the different biochemical processes (including hydrolysis, acidification and methanogenesis) of sludge AD, the microbial communities in the control and ZVI + CH-treated reactors were analyzed. Figure 7 depicts the relationships among microbial OTUs between the two reactors. There were 1577 of the same OTUs detected in both reactors, while 562 and 285 unique OTUs were present in the control and ZVI + CH-treated reactors, respectively. These results indicate that the microbial community structures varied significantly after the combined treatment. The genus-level distribution results are depicted in Figure 8. It was observed that several functional genera, including *norank_f__Bacteroidetes_vadinHA17*, *Hyphomicrobium*, *norank_f__Anaerolineaceae*, *Longilinea*, *Clostridium*, *Mycobacterium*, *Romboutsia*, *Sedimentibacter*, *Methanosarcina*, *Methanospirillum*, and *Methanosaeta*, existed in both reactors. However, their abundances showed distinct differences with or without the ZVI + CH treatment. For instance, the abundances of *norank_f__Bacteroidetes_vadinHA17* and *Hyphomicrobium*, two typical hydrolytic genera widely detected in different sludge AD reactors [46,47], were obviously enhanced from 0.95% and 1.17% in the control reactor to 2.1% and 1.89% in the ZVI + CH-treated reactor, respectively. The results suggest that

the ZVI + CH treatment is beneficial to the growth of hydrolytic microbes, because more AD substrates were degraded in this situation compared with the control group (Figure 4). The genus *norank_f__Anaerolineaceae* is a VFA producer that can utilize different kinds of carbohydrates as substrates [48], and its abundance was notably enhanced from 1.53% to 3.14% with the ZVI + CH treatment. Moreover, *norank_f__Anaerolineaceae* was also found to be a typical microbe involved in DIET [49]; thus, its enhanced abundance was undoubtedly beneficial to the AD reaction under the ZVI + CH condition. *Clostridium* is known as a VFA producer that can degrade macromolecular cellulose and has the potential to cooperate with electro-active methanogens via DIET [38,50]. Its abundance increased from 1.12% in the control to 1.75% in the co-treatment reactor. The genus *Sedimentibacter* is also an acidogenic bacterium that mainly utilizes amino acids and pyruvate as substrates, and its abundance enhanced from 0.21% to 0.95% after the ZVI + CH treatment. In addition, several other acidogens detected in this study were all enriched under the ZVI + CH condition (Figure 8).

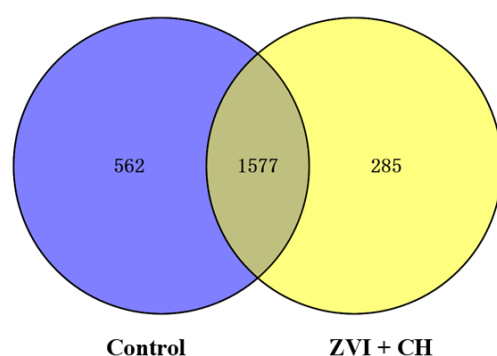


Figure 7. Venn diagram of microbial OTUs distribution between the control and ZVI + CH-treated reactors.

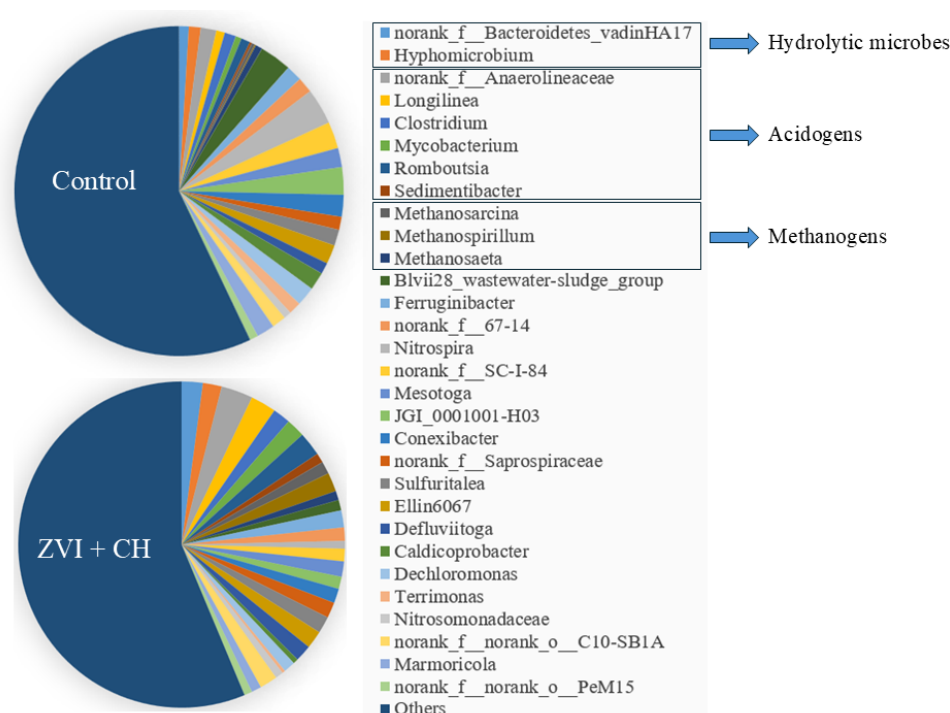


Figure 8. The microbial community structure in the control and ZVI + CH-treated reactors at genus level after 12 days of AD.

Except for hydrolytic and acidogenic genera, three methanogenic genera were found in both reactors, i.e., *Methanosarcina*, *Methanospirillum*, and *Methanosaeta*. Among them, *Methanospirillum* is a hydrogenotrophic methanogen that utilizes hydrogen as the substrate for methane generation, and its abundance was markedly enhanced from 0.11% to 1.87% after the ZVI + CH treatment. The genus *Methanosaeta*, an acetoclastic methanogen which utilizes acetic acid as the only substrate for methane production, was also enriched after ZVI + CH treatment, with abundances of 0.68% and 0.91% in the control and co-treatment reactors, respectively. *Methanosarcina* is a versatile methanogen that can adopt both hydrogen and acetic acid for methane production, and its abundance markedly increased from 0.11% to 1.87% after the ZVI + CH treatment. These results indicate that both hydrogenotrophic and acetoclastic methanogens were enriched by the ZVI + CH treatment. It should be noted that the facilitation of ZVI + CH on hydrogenotrophic methanogens was stronger than that on acetoclastic methanogens, as the total abundance of *Methanosarcina* and *Methanospirillum* increased by 7.1 times after the ZVI + CH treatment, while an increase rate of only 33.8% was observed in the abundance of *Methanosaeta* under the ZVI + CH condition. According to the previous literature [51], the exogenous hydrogen generated from the hydrogen evolution corrosion of ZVI can be directly utilized in the sludge AD process, which serves as a possible reason for the largely enhanced abundance of hydrogenotrophic methanogens produced by the ZVI + CH treatment in this study. According to above analysis, all functional microbes, including hydrolytic microbes, acidogens, and methanogens, were enriched when treated with ZVI + CH, as the total abundance observably increased from 8.41% to 20.58% after the combined treatment.

3.6. Overall Understanding

Though CH has been frequently proved to be valid for reinforcing sludge AD through high-efficiency hydrolysis [15,16], the increase rate in methane production is generally restricted to certain levels, as CH can inhibit the activities of methanogens, especially at high CH dosages. In this study, ZVI was introduced into the CH-pretreated sludge AD system to alleviate the adverse effect of CH on methanogens and thereby synergistically enhance methane production. The experimental results showed that both ZVI and CH effectively enhanced the methane yield from sludge AD, and their combination achieved a synergistic effect. The optimal condition for ZVI + CH treatment was 5 g/L of ZVI plus 0.12 g/g VSS of CH, and the corresponding methane yield was 275.8 mL/g VSS, significantly surpassing that achieved by the solo ZVI and solo CH treatments. To evaluate the effectiveness of the ZVI + CH method, the experimental results were compared with those of other CH-based methods reported in the previous literature [14,15,41,52,53]. As shown in Table 4, the increased rate of methane yielded by the ZVI + CH treatment in this work not only surpassed that of the solo CH treatment methods but also reached the maximum value among all combined methods, indicating that the ZVI-assisted CH method has technological superiority in sludge treatment.

Table 4. Comparison of methane increase rates between the ZVI + CH and other CH-based methods.

Methods	Treatment Condition		AD Con- dition	Methane Production		Increase Rate	Ref.
	CH Dosage	Other		Control	Treated		
Solo CH	0.24 g/g VS	-	37 °C	241.2 mL/g VS	385.1 mL/g VS	59.7%	[14]
Solo CH	0.05 g/g TS	-	52 °C	289.2 mL/g VS	362.2 mL/g VS	25.2%	[52]
CH + Freezing	0.075 g/g VSS	Freezing (−20 °C, 8 h)	35 °C	169.3 mL/g VSS	274.4 mL/g VSS	62.1%	[15]
CH + US	0.08 g/g VSS	US (1.0 W/mL, 10 min)	35 °C	168.2 mL/g VSS	263.4 mL/g VSS	56.6%	[41]
CH + TH	0.08 g/g VSS	TH (70 °C, 1 h)	35 °C	163.5 mL/g VSS	260.1 mL/g VSS	59.1%	[53]
ZVI + CH	0.12 g/g VSS	ZVI dosage of 5 g/L	35 °C	152.0 mL/g VSS	275.8 mL/g VSS	81.5%	This study

US—ultrasound; TH—thermal hydrolysis.

For the mechanism analysis, the kinetic model simulation indicated that both the methane production potential and rate were synergistically promoted by the ZVI + CH treatment, and the increase rates were 82.6% and 105.5%, respectively. By detecting the concentrations of typical organic matter after AD in different reactors, it was found that more organic matter (especially for easily biodegradable protein and polysaccharide) was degraded under the co-treatment condition, which is a key factor contributing to the increased methane yield. As important intermediate products in sludge AD, VFAs were largely generated in the initial stage under the ZVI + CH condition, and the maximum concentration was higher than those achieved by solo ZVI and solo CH. More importantly, the VFA concentration in the ZVI + CH reactor decreased and became lower than that in the solo CH reactor after 5 days of AD, suggesting that the assistance of ZVI reinforced the consumption of VFAs, thereby avoiding their accumulation. According to the results for the ETS and coenzyme F₄₂₀ activities, the ZVI + CH treatment not only enhanced the bioactivity of anaerobes but also relieved the CH-induced inhibition of methanogens. The microbial community analysis showed that all functional microbes, including hydrolytic microbes (e.g., *Hyphomicrobium*), acidogens (e.g., *Clostridium* and *Sedimentibacter*), and methanogens (e.g., *Methanosarcina* and *Methanospirillum*), were enriched under the ZVI + CH condition, and the total abundances were 8.41% and 20.58% in the control and ZVI + CH-treated reactors, respectively.

4. Conclusions

This study adopted ZVI to assist CH for sludge treatment to synergistically enhance AD performance. The experimental results showed that the maximal methane yield of 275.8 mL/g VSS was obtained when ZVI and CH dosages were 5 g/L and 0.12 g/g VSS, respectively, significantly higher than those of the solo ZVI or solo CH treatments. The mechanism analysis indicated that the ZVI + CH treatment reinforced the degradation of the organic matter in the sludge, especially the easily biodegradable proteins and polysaccharides. The enzyme activity analysis suggested that the ZVI + CH treatment promoted the bioactivity of anaerobes, and the suppression of methanogens by CH was eliminated in the presence of ZVI. The microbial community analysis illustrated that all functional microbes involved in sludge AD were enriched under the ZVI + CH condition.

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