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Algae biochar enhanced methanogenesis by enriching specific methanogens at low inoculation ratio during sludge anaerobic digestion

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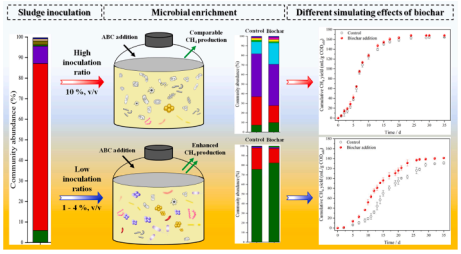
HIGHLIGHTS

- The effects of algae biochar at different inoculation ratios were investigated.
- Biochar enhanced methanogenesis at the lower ratios but not at the higher ratio.
- Methanogenic improvements at low ratios were not due to initial OLR increment.
- Microbial community structures at low ratios were all significantly changed.
- Biochar addition at low ratios was more favorable for *Methanosarcina* enrichment.

GRAPHICAL ABSTRACT



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ABSTRACT

Carbon materials are promising in improving the performance of anaerobic digestion, however, interactive mechanisms between the carbon-based enhancement and operating parameters remained unclear. Using anaerobic digested sludge as inoculum, the effects of Taihu blue algae biochar (ABC) on methanogenesis at different inoculation ratios were investigated during sludge anaerobic digestion. Results showed that ABC enhanced methane productions at the lower inoculation ratios (4% and 1%, v/v), but not at the higher ratio (10%, v/v). Mechanism analysis demonstrated methanogenic improvements at the lower inoculation ratios were not owing to initial organic loading rate increments. Otherwise, ABC addition at the lower inoculation ratios were more favorable for the enrichment of *Methanosarcina* than the higher ratio, which might be benefit for methanogenesis through directed interspecies electron transfer. Thus, for the improvement of sludge anaerobic digestion, the microbial enrichments at different inoculation ratios would be more important than the merely biochar addition.

1. Introduction

As the inevitable byproduct of biological wastewater treatment, sewage sludge is an important source of secondary pollution as well as resource recovery for human society (Appels et al., 2011; Batstone and Virdis, 2014). Anaerobic digestion is a reliable and sustainable

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technology for sewage sludge treatment, while the efficiency of anaerobic methanogenesis is often limited by relatively slow syntrophic metabolism between acidogens and methanogens (Barua and Dhar, 2017; Gahlot et al., 2020; Li et al., 2019; Xu et al., 2019). Recently investigators have proposed that directed interspecies electron transfer (DIET) was a new efficient mechanism for interspecies electron exchange during anaerobic digestion (Rotaru et al., 2014a, 2014b). Moreover, supplementations of carbon materials (e.g. biochar, granular activated carbon, graphene, carbon nanotube, etc.) were reported to facilitate electron exchange and thus enhancing syntrophic methane production in anaerobic system. Consequently, the past decade has seen the rapid development of carbon-based strategies for waste reduction and energy recovery through anaerobic digestion (Lei et al., 2019; Lin et al., 2018; Wu et al., 2019; Zhang et al., 2019; Zhao et al., 2017b).

Generally, DIET is based on applying carbon material as electron conduit between syntrophic partners. Therefore, major characteristics that related to electron exchange, like conductivity (Liu et al., 2012), redox property or electron-donating capacity (Cruz Viggi et al., 2017; Wang et al., 2020; Zhang et al., 2018) and surface functional groups (Ren et al., 2020; Yuan et al., 2018), were successively considered to be the key factors to methanogenic improvement by DIET. Otherwise, some researchers believed that the establishment of the DIET-based syntrophic metabolism should be the primary target for methanogenic improvement. For instance, both Li et al. (2020a) and Zhao et al. (Zhao et al., 2016a, 2017a, 2020) have found that the abundance of *Methanosarcina* or *Methanosaeta* species was evidently increased after the anaerobic digesters were feed with ethanol in the starting stage, which also confirmed the establishment of DIET-based strategy for methanogenic improvement during anaerobic digestion. The microbial aggregation of many functional microorganisms (e.g. granular sludge) was also proposed to be beneficial to the enhancement of microbial interspecies relationships (Zhang and Jiang, 2019; Zhang et al., 2021). However, for a given biotechnological process like sewage sludge anaerobic digestion, it is still uncertain that the carbon-based strategies for methanogenic enhancement should be focused on the carbon properties or the enrichment of electron syntrophic couples.

Moreover, the function of DIET was found varied with the operational conditions, like temperature (Lin et al., 2018), mixing speed (Kariyama et al., 2018) as well as ammonium concentration (Yan et al., 2019, 2020). Additionally, Ren et al. (2020) have declared that after the reactors were started with different initial organic loading rates (OLR) in batch operations, although the addition of sludge hydrochar was proved to improve methanogenesis through DIET at the higher OLR operations, no significant differences were observed in the lower OLR operations with or without hydrochar. To the best of our knowledge, DIET was accepted as a more efficient mechanism for syntrophic methane production, especially under high organic loading conditions (Lei et al., 2019; Wang et al., 2019; Zhao et al., 2017c). However, it should be noted that both the variation of inoculation ratios and substrate concentrations could lead to initial OLR change during batch operations, but little is known about the effects of this two OLR regulation methods on methanogenesis during anaerobic digestion. Moreover, in addition to the OLR differences, both the metabolic pathways and the microbial community structures would be varied with different inoculation ratios in batch operations (Lü et al., 2016, 2019). Therefore, it can be assumed that the carbon-based enhancement for methanogenesis might be affected by the metabolic changes under different inoculation ratios, while this process has yet to be fully investigated.

In this study, biochar was derived from Taihu blue algae biomass, considering that the combination of pyrolysis and anaerobic digestion offers an opportunity for sustainable management of harmful blue algae and sewage sludge (Monlau et al., 2016). Moreover, anaerobic digested sludge was taken as inoculum, then the effects of algae biochar (ABC) at different inoculation ratios were investigated during batch anaerobic digestions of sludge hydrolysate. The objectives of this study included (1) to explore the simulating effects of ABC addition in the anaerobic digestion of sewage sludge at different inoculation ratios; (2) to explore the effects of ABC addition in the anaerobic digestion of sewage sludge at different initial substrate concentrations; (3) to explain the mechanisms that associated with the ABC-mediated simulating effects at different inoculation ratios; (4) to clarify the prerequisites for the improvement of sludge anaerobic digestion through ABC addition. The findings of this study provided a novel understanding of interactions between the operating parameters and the carbon-based enhancement during methanogenic process, which would be benefit for waste reduction and energy recovery through anaerobic digestion.

2. Materials and methods

2.1. Preparation of feedstock, inoculum and algae biochar

Dewatered sludge (mixed primary sludge and activated sludge) was collected from Xincheng wastewater treatment plant (Wuxi, China) and stored at 4 °C. Main characteristics of the dewatered sludge were as follows: total solids (TS, w/w) of 17.6 ± 1.6% and volatile solids/total solids (VS/TS, w/w) of 50.1 ± 2.8%. The dewatered sludge was diluted with distilled water to the pre-set concentration before use. As reported, liquid anaerobic digestion was operated with sludge hydrolysate after pretreatment as substrate, which has been proved to have lower operating cost and higher value returns (Lu et al., 2020). Thus, the diluted sludge was thermo-alkaline pretreated (pH = 10, 105 °C, 24 h), then sludge hydrolysate was obtained by solids-liquid separation (5000 rpm, 10 min) and finally conducted to anaerobic digestion in this study.

The inoculum sludge was collected from an anaerobic digester that operated semi-continuously at mesophilic temperature (35 ± 2 °C). Feeding with the same sludge from Xincheng, the reactor has been operated steadily for >400 days. Other detailed information about the inoculum could be found in a previous study (Jiang et al., 2021).

Blue algae biomass was freshly collected from the blue algae refloating sites alongside Taihu Lake of Wuxi city, China, in which the dominated community was *Microcystis*. After cold drying, the obtained algae powder was subjected to pyrolysis in a nitrogen-protected tube furnace (Shengli Instruments, China). The pyrolysis temperature was initially heated up to 450 °C at the rate of 10 °C/min and then maintained at 450 °C for 2 h. Finally, the obtained algae biochar (ABC) was crushed and sieved to a particular size of 60-mesh, then ABC was sealed and stored at room temperature before use. As for the characterization of ABC in this study, the pH was 7.2 ± 0.1; the average particle size of ABC was 100–300 μm; the specific surface area was 145.6 ± 39 m^2/g, the iodine value and phenol adsorption value were 289.8 ± 87 mg/g and 101.2 ± 26 mg/g, respectively.

2.2. Setting for anaerobic digestion at different inoculation ratios

The effects of ABC on the anaerobic digestion with different inoculation ratios were investigated in batch operations. The experiments were conducted in eight customized glass reactors (1.2 L) with working volume of 800 mL. Sludge hydrolysate was prepared from the diluted sludge with the solid concentration of 4% (w/w). Each reactor contained sludge hydrolysate 720 mL, trace element solution, 8 mL; vitamin solution, 8 mL. The composition of the trace element solution and vitamin solution were described in a previous study (Zhao et al., 2016a). In order to set different inoculation ratios, the inoculum to substrate ratio based on volume was set to 10%, 4%, 1% and 0, respectively. ABC was added to each digester at the dosage of 10 g/L, and the reactors that without ABC addition at each inoculation ratio were set as the control groups. pH of all the mixtures was initial adjusted to 7.0 ± 0.5 by 3 mol/L hydrochloric acid and sodium hydroxide, and there was no pH adjustment during the sludge anaerobic digestion process. Before sealing, all the reactors were flushed with high purity nitrogen gas for >30 min to maintain anaerobic condition. Then all reactors were placed in a greenhouse and the temperature was conducted at 35 ± 1 °C, the

mixture in each reactor were stirred with an agitator at the speed of 120 rpm continuously. Biogas samples were collected by gasbags that installed on the top of each digester. Liquid samples were taken from vials following reaction for analysis. In order to confirm the methanogenic performances at different inoculation ratios, repetitive experiments have been arranged after the first batch anaerobic digestion in this study.

2.3. Setting for anaerobic digestion with different initial substrate concentrations

The effects of ABC on the anaerobic digestion at different initial organic loading conditions were investigated in batch experiments. Notably, anaerobic digestions at different initial organic loading conditions were arranged by different initial substrate concentrations. Specifically, dewatered sludge was diluted to the total solid concentration of 2%, 4%, 6% and 8% (w/w), respectively, and sludge hydrolysate with different chemical oxygen demand (COD) concentrations were prepared by this different diluted sludge after thermo-alkaline pretreatment and solids-liquid separation. Then different sludge hydrolysates mentioned above were used as substrates for the anaerobic reactors that named as B1 to B4, respectively. The inoculation ratio based on volume was set to 10% for each reactor, and therefore the digestion reactions with different initial organic loading conditions were settled. ABC was added to each digester at the same dosage of 10 g/L. Other details were in consistent with the description in section 2.1. The repetitive experiments were also arranged after the first batch anaerobic digestion in this study.

2.4. Microbial community analysis

In order to further explore the potential mechanism that might be involved in the different simulating effects at different inoculation ratios, microbial community structure was analyzed based on the high-throughput sequencing of 16S rRNA genes before and after anaerobic digestions. Specifically, sample 'Z' was obtained from the inoculum digester; moreover, microbial samples A2, A4 and A6 were collected from the experimental groups with 1%, 4% and 10% inoculation ratios (v/v) at end of the batch operations, respectively; samples A1, A3 and A5 were control groups for the above three groups, respectively. After total DNA extraction, DNA concentration and purity were determined by a micro volume spectrophotometer (NanoDrop 2000, USA). 16S rRNA gene fragments were amplified via the polymerase chain reaction (PCR) with the following primer sets: 338F-806R and 524F-Arch958 modR. The PCR products were purified, quantified and sequenced by Illumina Miseq platform, Shanghai Majorbio Bio-pharm Technology Co., Ltd (China). Sequences were placed into various operational taxonomic units (OTU) and data were analyzed on the free online platform of Majorbio Cloud Platform (http://www.majorbio.com).

2.5. Methane production modeling analysis

The modified Gompertz model was used to simulate the methane production performance in the batch digestion assays (Nopharatana et al., 2007):

$$P_{(t)} = P_m \cdot exp\left\{ -exp\left[\frac{R_m \cdot e}{P_m}(\lambda - t) + 1\right]\right\} \quad (1)$$

where: $P_{(t)}$ is the cumulative methane production at time t, mL/g COD; P_m is the maximum methane production potential, mL/g COD; R_m is the maximum methane production rate (MPR), mL/g CODd; λ is the lag phase time, d; t is the duration time, d; e is constant (2.71). The methane production curve and the relevant parameters were simulated using Origin Pro 9.0 (Origin Lab Corporation, MA, US).

2.6. Chemical analysis

The COD, total solids (TS) and volatile solids (VS) were conducted according to the methods described in a previous study (Ma et al., 2016). The gas component was analyzed by a gas chromatography (GC9790II, FuLi, China) with a thermal conductivity detector and a stainless steel packed column (AE.TDX-01, China). The volume of biogas was measured by displacement of saturated aqueous sodium chloride in a graduated measuring cylinder. The gas volume was calibrated to standard conditions (273 K, 1 atm) after measurement. The volatile fatty acids (VFAs) concentration was detected by a gas chromatography (GC-2010, Japan) equipped with a flame ionization detector and a fused-silica capillary column (PEG-20M, China).

2.7. Statistical analysis

The one-way analysis of variance was used to test the significance of the results, and $p < 0.05$ was considered as a statistical criterion.

3. Results and discussion

3.1. Effects of algae biochar on methane productions at different inoculation ratios

In this study, the anaerobic reactors were inoculated with different amounts of inoculum sludge at the starting period, and then methane productions were monitored throughout the anaerobic digestion processes. As shown in Fig. 1a, there were no significant differences between methane productions with and without ABC addition at the inoculation ratio of 10% (v/v) ($p > 0.05$). In the control and ABC-supplemented reactors, the observed lag phases (2.8 d and 2.4 d) and the cumulative methane yields (161.1 mL/g COD and 166.4 mL/g COD) were statistically similar (Fig. 1a). However, for methane productions at the lower inoculation ratios of 4% and 1% (v/v), ABC addition (10 g/L) enhanced methanogenic processes comparing to the control groups (Fig. 1b, c). For example, comparing to the controls, the reduced lag phase (41.6% and 44.3%), the increased methane production rate (12.2% and 17.5%) and methane production potential (7.4% and 9.6%) were observed at inoculation ratios of 4% and 1% (v/v) with ABC addition (shown in supplementary material). Moreover, no methane production was observed without seed sludge throughout the digestion processes (Fig. 1d), indicating that sludge inoculation has played a key role in the sludge hydrolysate digestion.

Furthermore, the observed kinetic parameters and COD removals from the methanogenic performance with ABC addition were compared to the control groups, then influence of ABC addition on mathematical constants were simulated to the inoculation ratios. As shown in Fig. 2, it was obvious that excepting for the groups without inoculum (Fig. 1d), the methanogenic improvements by ABC were all negatively correlated to the initial inoculation ratios (Fig. 2a, b, c). Specifically, the improvement of methane production potential (P_m) and methane production rate (R_m), as well as the reduction of lag phase (λ) for the methanogenic performances at the inoculation ratios of 4% and 1% were all higher than that at the 10% ratio, which also confirmed that ABC enhanced methane productions at the lower inoculation ratios (4% and 1%, v/v), but not at the higher ratio (10%, v/v). As to the substrate COD removal, comparing to the controls, the substrate COD removal rates were improved after ABC addition, especially at the lower inoculation ratios. For instance, the enhancement of COD removal rates by ABC were 26.1 ± 5.4% and 25.9 ± 2.1% at 4% and 1% (v/v) inoculation ratios after digestion, respectively, but that at the 10% ratio (v/v) was only 0.7 ± 0.2% comparing to the groups without ABC addition (Fig. 2d). Clearly, ABC addition at the lower inoculation ratios (4% and 1%, v/v) were more conducive to the degradation of organic substrates than the higher ratio (10%, v/v), which also explained the methanogenic enhancement by ABC at the lower inoculation ratios. Overall, these

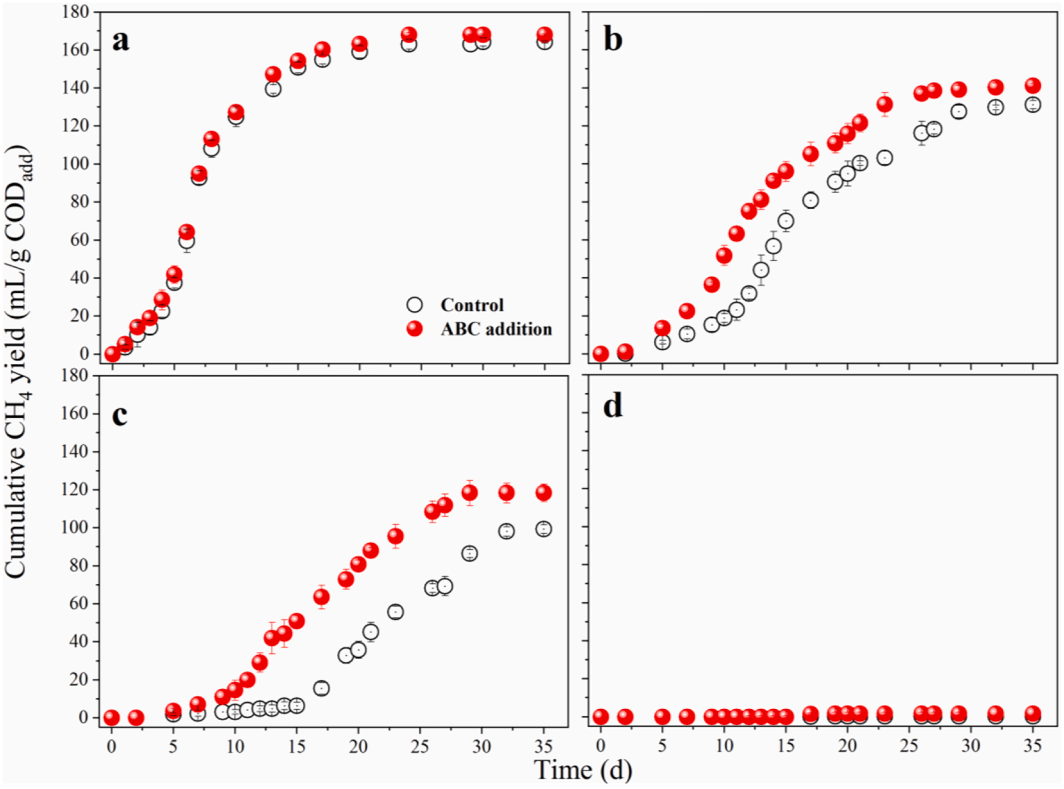

Fig. 1. Influences of ABC addition on methane productions at different inoculation ratios (v/v): (a) 10%; (b) 4%; (c) 1%; (d) 0%.

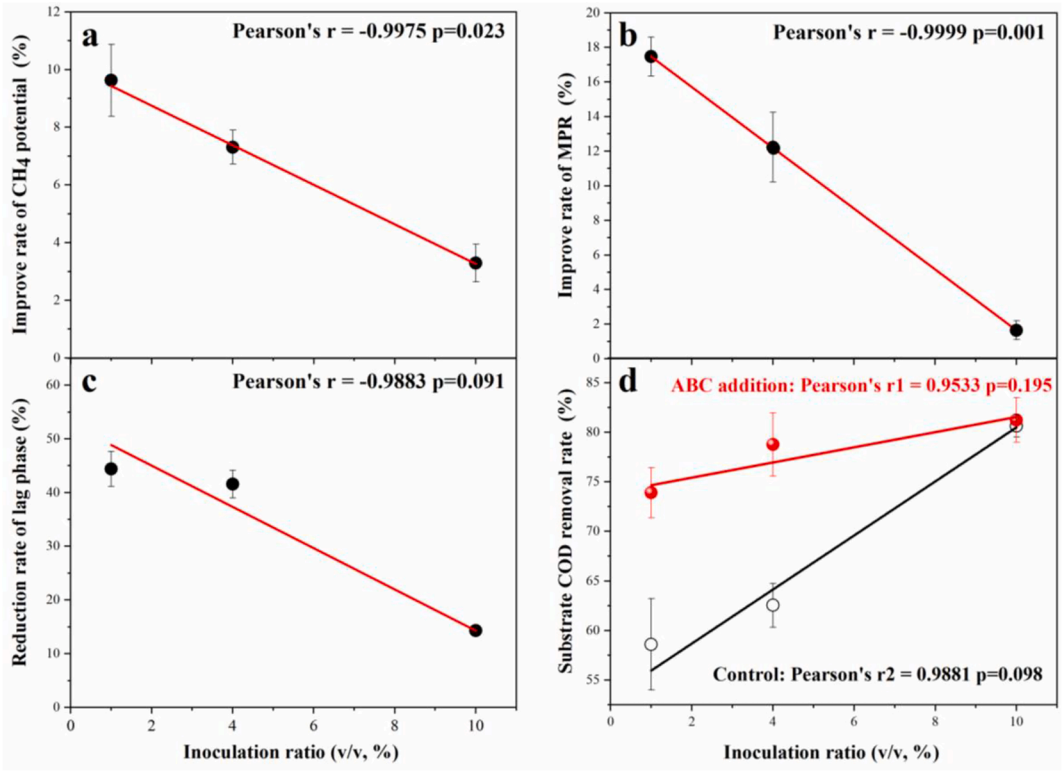

Fig. 2. Correlations between the inoculation ratios and the influences of ABC addition on mathematical constants (a: methane production potential, Pm; b: methane production rate, MPR; c: lag phase, λ), substrate COD removal rates (d) in sludge anaerobic digestion.

results suggested that the addition of ABC enhanced methanogenic process at the lower inoculation ratios (4% and 1%, v/v), but not at the higher inoculation ratio (10%, v/v).

As reported, the variation of inoculation concentration was believed to result in different initial organic loading conditions in batch operations, and the addition of biochar was proposed to improve methanogenic process through DIET at the higher OLR operations (Ren et al., 2020; Xu et al., 2018). This mainly because a common understanding is that DIET facilitated by conductive materials (like biochar, granular activated carbon, etc.) is a more efficient mechanism for syntrophic methane production, especially under high organic loading conditions. For example, Zhao et al (2016b) have found that the effectiveness of the up-flow anaerobic sludge blanket (UASB) reactor with conductive material addition was significantly increased over the control reactor when the OLR were increased. In addition, it was widely accepted that the common working mode for syntrophic methanogenesis was interspecies hydrogen transfer (IHT), in which H_2 served as the electron carrier (Stams and Plugge, 2009). However, the efficiency of IHT is relatively low due to the diffusion limitation of electron carriers. Zhao et al (2017c) have proposed that the interspecies electron exchange could shift from IHT to the more efficient DIET mechanism in presence of carbon cloth, which helped to maintain syntrophic metabolism and resist acidic shocks during anaerobic digestions.

In many cases, the increase of OLR was considered to be an important factor that triggered the carbon-based DIET syntrophic relationships between acidogens and archaea during anaerobic digestion (Lei et al., 2019; Wang et al., 2019). However, it should be noted that the OLR variation could be obtained by changing inoculation ratio as well as initial substrate concentration in batch operations; moreover, in addition to the OLR differences, both the metabolic pathways and the microbial community structures would be varied with different inoculation ratios (Li et al., 2020b; Kawai et al., 2014). Whether or not the different simulating effects of ABC between high and low inoculation ratios (Fig. 1) were due to the initial OLR variations remained to be further investigated.

3.2. *Effects of algae biochar on anaerobic digestions with different initial substrate concentrations*

Sludge hydrolysates that prepared from waste activated sludge with different solid concentrations were used as substrates for anaerobic digestion, then the effect of ABC addition (10 g/L) on methane production process under different initial organic loading conditions were investigated. The initial substrate concentrations in reactors B1 to B4 were 9172.1 ± 673.2, 18926.5 ± 1008.5, 26594 ± 1085.6 and 34625.9 ± 1424.5 mg COD/L, respectively. Therefore, different initial organic loading conditions of anaerobic have been arranged in reactors B1 to B4 at the same inoculation ratio (10%, v/v).

The methanogenic performances of anaerobic digestion with ABC addition at different organic loading conditions were shown in Fig. 3. Obviously, for cumulative methane production, the increase of initial OLRs improved the cumulative methane productions, and the final methane productions of B1 to B4 were 817.3 ± 57.3, 1761.3 ± 112.3, 2391.1 ± 132.6 and 3176.2 ± 185.6 mL, respectively (Fig. 3a). More organics were converted to methane at the higher OLR conditions, which explained the methane productions were increased with the OLR increment. Moreover, the accumulation of metabolites (e.g. ammonium) would cause the pH variation during sludge hydrolysate digestion, and the increased substrate conversion also resulted in higher pH values under the higher OLR conditions. For example, the pH values in reactor B1 to B4 were finally stabilized at 7.63 ± 0.11, 7.89 ± 0.15, 7.95 ± 0.08 and 7.99 ± 0.13, respectively (Fig. 3c).

However, the increase of initial OLRs did not improve the cumulative methane yields per unit of substrate COD added (COD_{add}). For the experimental groups, methane yields of B1 to B4 were 123.8 ± 4.7, 125.5 ± 9.5, 124.9 ± 6.4 and 127.4 ± 3.2 mL/g COD_{add}, respectively

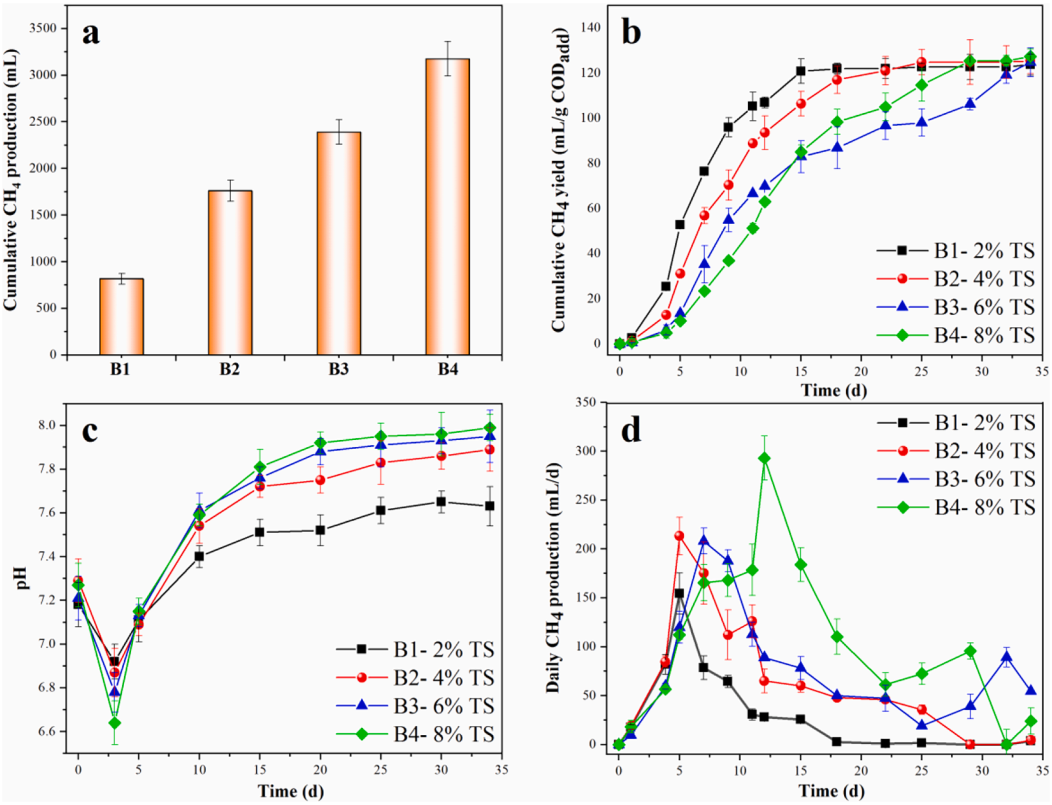

Fig. 3. Influences of initial substrate concentration on methane productions with ABC addition: (a) cumulative methane productions; (b) cumulative methane yield per unit of COD_{add}; (c) pH variations; (d) daily methane productions. 'TS' refers to 'total solids'.

(Fig. 3b), no significant difference was observed between the methane yields of four groups ($p > 0.05$). Moreover, it was also found that ABC addition did not accelerate methanogenic process under the higher organic loading conditions (Fig. 3b). On the contrary, due to the prolonged substrate conversion process, methane production rate at the higher organic loading conditions were reduced comparing to the lower OLRs digestions. For example, the peak times of daily methane production in the reactor B1 to B4 were 5.1, 5.5, 6.5 and 12.6 days, respectively (Fig. 3d). In addition, as the major intermediate for methanogenesis, VFAs of the reactor B3 and B4 were found to be consumed at a significantly lower rate than the reactor B1 and B2 (shown in supplementary material), which also indicating that substrate conversion process were prolonged in the higher organic loading groups (B3 and B4).

Obviously, after been inoculated at the ratio of 10% (v/v), the increments of initial substrate concentration did not help to improve methanogenic performance even in presence of ABC. Notably, although the calculated OLRs of reactors B3 and B4 were comparable to the reactors with inoculation ratio of 4% and 1% (v/v) in section 3.1 (Fig. 1b, c), respectively, neither methane yield increment nor methanogenic acceleration was observed under the higher organic loading condition (Fig. 3b). On the contrary, methanogenic processes were prolonged by the elevated substrate concentrations, which might lead to the overall methane production rate decrements during sludge anaerobic digestions (Fig. 3b, d). These results also indicated that methanogenic enhancements by ABC at the lower inoculation ratios (4% and 1%, v/v) might not attributed to the initial OLR increments.

Currently, the DIET mechanism is still considered to be an important mechanism for the enhanced methanogenic performance with biochar addition (Lei et al., 2019; Zhao et al., 2017b). However, relationships between the OLR increment and the DIET syntrophic relationships should be reconsidered, since no methanogenic improvement was observed at the higher organic loading conditions with ABC addition in this study. According to Ren et al. (2020), the presence of abundant bacteria and archaea at high inoculation concentrations might result in the fast consumption of substrates, which also suggesting that the variations of microbial community at different inoculation concentrations should be evaluated. It is worth noting that in addition to the initial OLR increment, the metabolic pathways and the microbial community would be affected by inoculation ratio as well. This is because anaerobes are strict with their environment (Lv et al., 2019), especially with the presence of biochar, both the microbial community structures and the metabolic pathways would be affected by the change of initial inoculation ratios, thus leading to the different effects on methane production.

3.3. *Effects of algae biochar on microbial community diversity at different inoculation ratios*

16S rRNA amplicon sequencing was used to explore the different effects of biochar addition on microbial diversity and community structure. A total of 265,230 high-quality bacterial and 183,920 archaeal sequences were obtained, which were clustered into 1591 and 53 OTUs respectively based on 97% sequence similarity. To visualize differences between the microbial communities in each reactor, principal co-ordinates analysis (PCoA) was calculated using weighted unifrac distance.

As shown in Fig. 4, both bacterial and archaeal communities showed a similar β-diversity pattern, that is microbial communities of the inoculum sludge were obviously different from that of the experimental groups (Fig. 4a, c), which indicating that the microbial community structures have changed significantly after anaerobic digestion. That was probably because the digestion substrate has been changed from

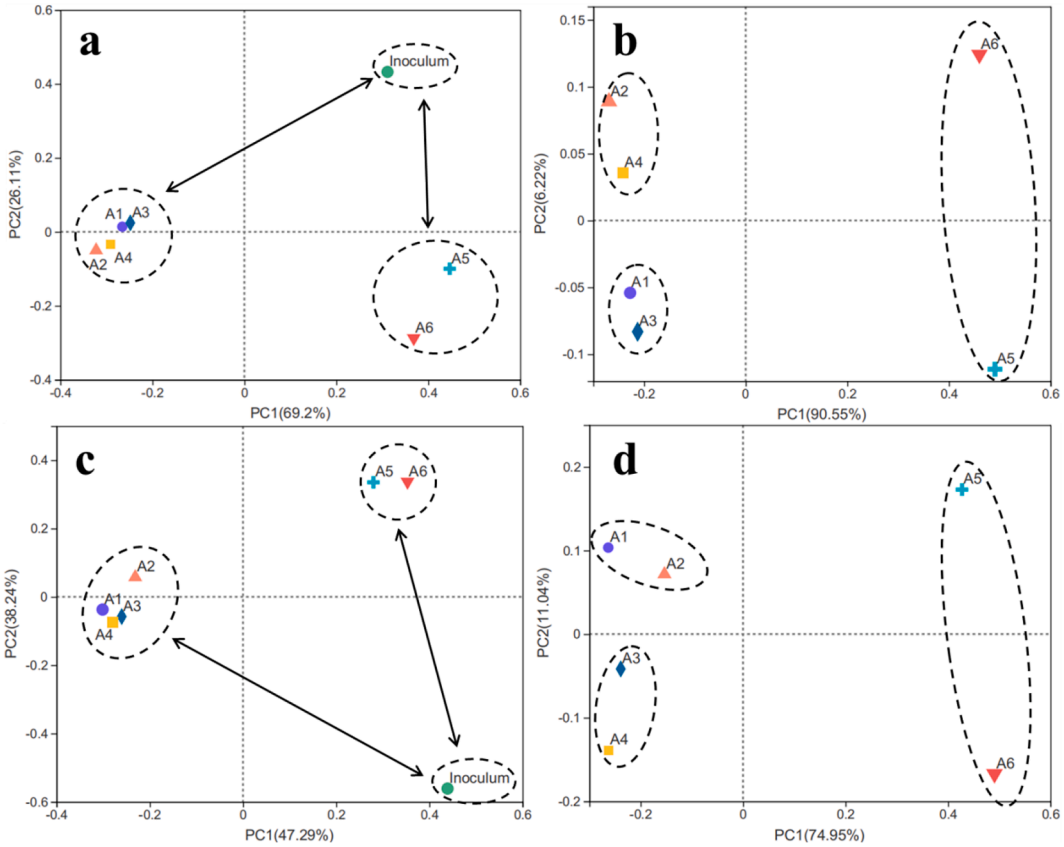

Fig. 4. Principal Coordinate Analysis of microbial community structure of archaea (a, b) and bacteria (c, d). Sample 'Inoculum' was the seeding sludge; samples A2, A4 and A6 were collected from the experimental groups with 1%, 4% and 10% inoculation ratios (v/v), respectively; samples A1, A3 and A5 were control groups for them, respectively.

thickened sludge to sludge hydrolysate in this study. Notably, in comparison with the difference between the inoculum sludge (sample 'Inoculum') and the experimental groups (sample A1 to A6), evident distances were observed between the higher inoculation ratio groups (A5 and A6) and the lower ratio groups (A1 to A4). These results clearly indicated that different microbial community structures have developed at different inoculation ratios. Moreover, it should be noted that among the lower inoculation ratio treatments (A1 to A4), the microbial communities of control groups (A1 and A3) were more similar, while significant differences between the ABC addition groups (A2 and A4) and the control groups were observed in both bacterial and archaeal community diversities under all three inoculation ratios (Fig. 4b, d). Hierarchical clustering analysis based on OTU weighted unifrac distance of archaea and bacteria also showed that A1 and A3 treatments could cluster together, while A2 and A4 samples could cluster together or form a single cluster (shown in supplementary material). Additionally, for archaeal community diversity (shown in supplementary material), the Shannoneven index and the Invsimpson index of the ABC addition groups (A2 and A4) were all reduced comparing to the control groups (A1 and A3).

Taken together, the above results therefore suggested that microbial communities showed unique structures and compositions based on

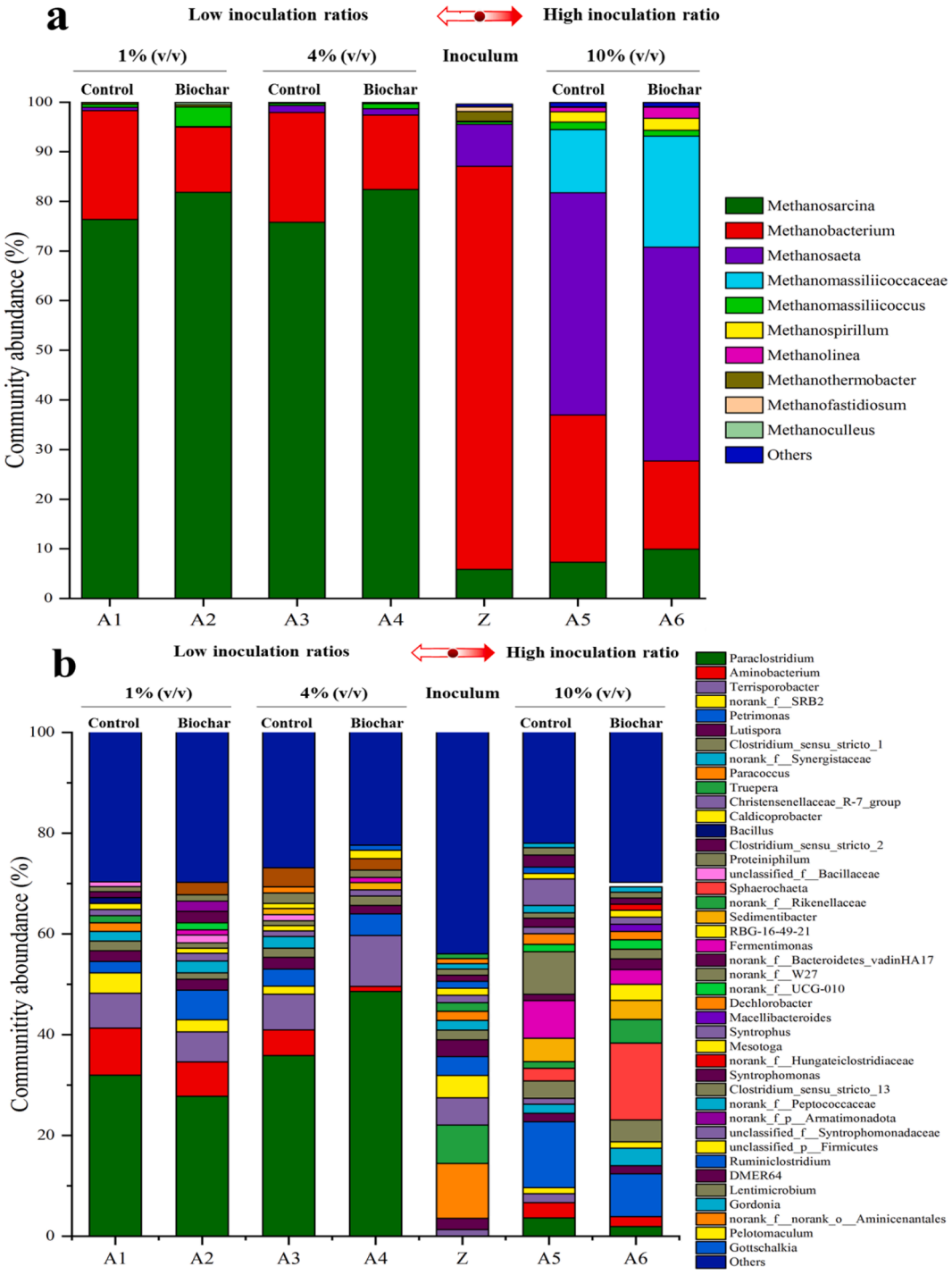

Fig. 5. Dynamic changes in the community composition of archaea (a) and bacteria (b) at the genus level. Genus level with relative abundance lower than 1% were classified into group 'others'.

inoculation ratios as well as ABC addition. Notably, the microbial communities, especially the archaeal communities, were greatly affected by ABC at the lower inoculation ratio groups. The archaeal community diversity was declined significantly after ABC addition, suggesting that ABC addition might have promoted the enrichment of some specific archaea at the lower inoculation ratios during sludge anaerobic digestion.

3.4. Effects of algae biochar on microbial community structure at different inoculation ratios

To clarify the impact of ABC on the composition of microbial communities, dominant bacteria and archaea at the phylum and genus level were compared before and after anaerobic digestion in this study. For archaea, three phyla including *Halobacterota*, *Euryarchaeota* and *Thermoplasmatota* were dominated in the experimental groups (A1 to A6). Specially, *Halobacterota* was the most dominant phylum in experimental groups with a relative abundance of 77.3% to 82.6%, while the dominant phylum in the inoculum sludge was *Euryarchaeota* (84.3%). Further genus-level studies showed that five archaea genera included *Methanosarcina, Methanobacterium, Methanosaeta, Methanomassiliicoccus* and *Methanothermobacter* were dominated in most of the samples (Fig. 5a). After anaerobic digestion, the relative abundances of the *Methanosarcina* were significantly increased from 5.9% in inoculum to 75.8% to 82.4% in samples A1 to A4. Notably, among the four treatments, the relative abundances of *Methanosarcina* in A2 and A4 treatments (81.8% and 82.4%) were significantly higher than that in the control group A1 and A3 (76.3% and 75.8%) ($p < 0.05$). Obviously, comparing to the controls, the enrichment of *Methanoscrina* species were significantly promoted by ABC at the lower inoculation ratios (4% and 1%, v/v).

Interestingly, different dominant archaea were observed in the higher inoculation ratio groups (A5 and A6), since the relative abundances of *Methanosaeta* were significantly increased from 8.5% in inoculum to 44.8% and 43.1% in A5 and A6 treatments, respectively, while that of *Methanoscrina* species were maintained at 7.3% and 9.9%, respectively (Fig. 5a). It was clear that *Methanosaeta*, not *Methanosarcina* species, were enriched at the higher inoculation ratio, which also confirmed the micorbial community diversity results in section 3.3.

For bacteria, the dominant phylums like *Firmicutes, Proteobacteria, Bacteroidota, Synergistota, Actinobacteriota,* and *Chloroflexi* were found in A1 to A6 treatments, accounting for 91.7% of the total sequence. These phyla are common in anaerobic fermentation reactors, which are closely related to the degradation of complex substrates (Dang et al., 2016; Zhao et al., 2017b). Moreover, Fig. 5(b) shows the genus level distributions of the bacterial community in both the inoculum and anaerobic digested samples. The dominant bacterial genera (relative abundance > 1%) were *Paraclostridium* (27.9% to 48.6%), *Terrisporobacter* (5.9% to 9.8%), *Aminobacterium* (1.1% to 9.3%) and *Petrimonas* (2.3% to 5.9%) in A1 to A4 treatments. However, more dominant bacterial genera were observed in the higher inoculation groups (A5 and A6), which also indicating that bacterial community at the higher inoculation (10%, v/v) was significantly different from that at the lower inoculation ratios (4% and 1%, v/v). Specially, the most dominated microorganisms (e.g. *Paraclostridium*) in this study has been proposed to be capable of maintain electron syntrophic relationships with methanogens in anaerobic digesters (Zhao et al., 2017b). Moreover, it should be noted that none of the above four bacterial genera were dominant in the inoculum sludge. Therefore, the results revealed the populations of

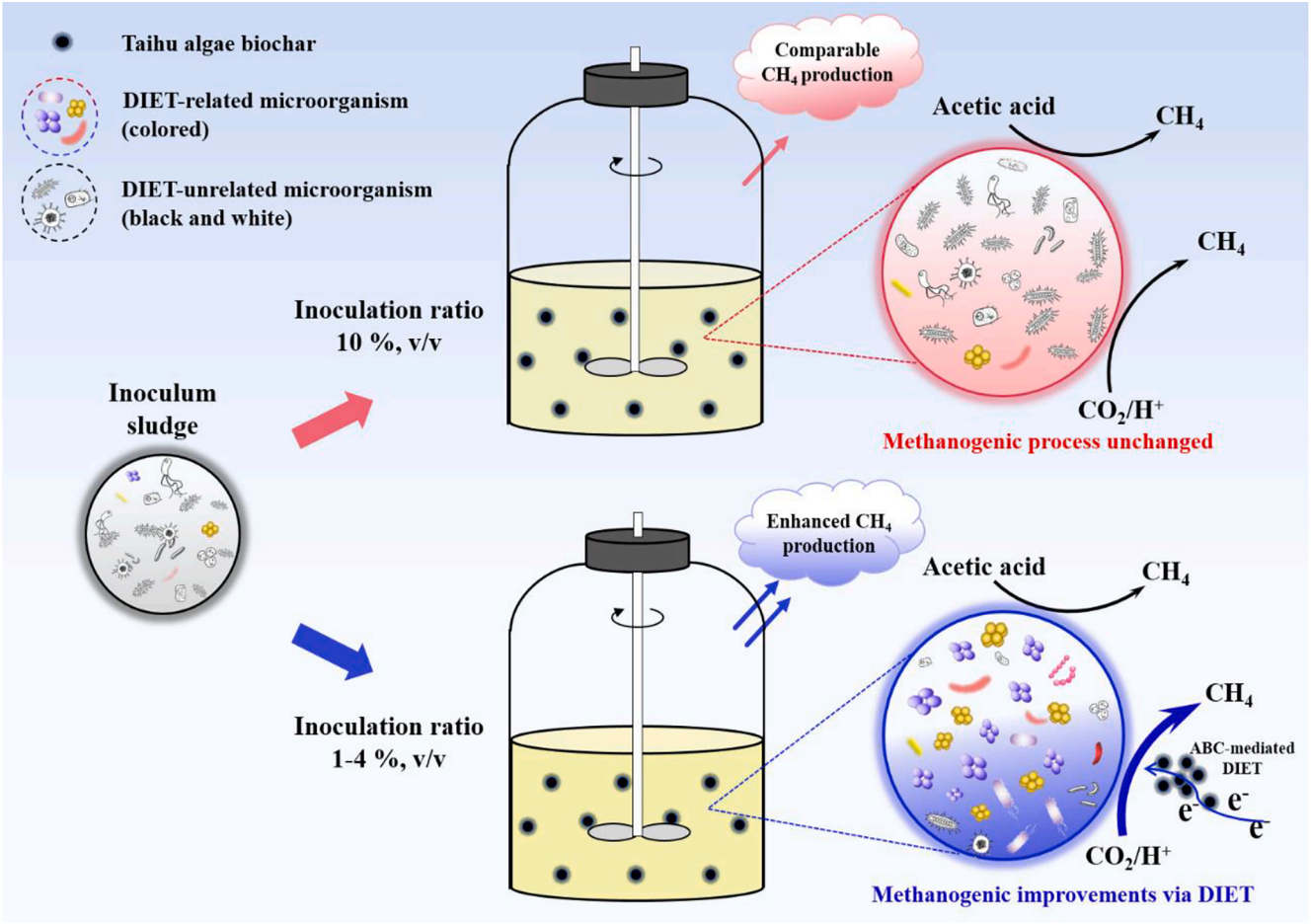

Fig. 6. Schematic diagram of potential methanogenic mechanism with Taihu algae biochar addition at different inoculation ratios during sludge anaerobic digestion.

biodegradation-associated microorganisms were increased during the anaerobic digestion of sludge hydrolysate, thus providing favorable conditions for organics degradation and syntrophic methane production with ABC addition.

3.5. Potential methanogenic mechanism with algae biochar addition at different inoculation ratios

As illustrated in Fig. 6, although the anaerobic reactors were inoculated with the same inoculum sludge, microbial community structures at the lower inoculation ratios (4% and 1%, v/v) were significantly different from that at the higher ratio (10%, v/v), and different dominated methanogens were enriched at different inoculation ratios. More importantly, comparing to the higher inoculation ratio, the addition of ABC at the lower inoculation ratios significantly promoted the enrichment of *Methanoscrina* species, which might be involved in the methanogenic improvements through DIET.

Up to now, the DIET-syntrophic partners for methane production were limited to few genuses that defined in pure cultures, like *Methanosarcina barkeri* (Rotaru et al., 2014a) or *Methanosaeta harundinacea* (Rotaru et al., 2014b). Notably, *Methanosarcina* species are the few methanogens that can perform both hydrogenotrophic and aceticlastic methanogenesis (De Vrieze et al., 2012). Although it is difficult to detect the electron transfer process directly in anaerobic digestion of complex substrates (Van Steendam et al., 2019), DIET was still considered to be a probable mechanism for the enhanced methanogenic performance with biochar addition (Ren et al., 2020; Wang et al., 2020; Wu et al., 2019; Xu et al., 2018), and the enrichment of electroactive genus, like *Methanosarcina* species, has been also proved to be crucial for the DIET-based syntrophic methanogenesis by many researchers (Dang et al., 2016, 2017; Lei et al., 2016; Yan et al., 2017; Zhang et al., 2020).

However, *Methanosarcina* species typically grow slowly on acetate since the conversion of acetate to methane yields little energy (Dang et al., 2016; De Vrieze et al., 2012). Moreover, for the sludge anaerobic digestion, *Methanoscrina* species that associated with DIET are often low in abundance (Fig. 5b). As a result, under the higher inoculation ratio (10%, v/v), the presence of abundant bacteria and archaea that unrelated to DIET have resulted in the fast consumption of substrates and intermediates for methanogenesis (shown in supplementary material), methanogens that involved in the syntrophic methane production (e.g. *Methanosarcina*) might be uncompetitive and therefore no differences in methanogenesis were observed even with ABC addition. As to the lower inoculation ratios (4% and 1%, v/v), the consumption of VFAs was significantly decelerated due to the reduction of inoculation ratios (shown in supplementary material), *Methanoscrina* might benefit from accepting the extra electrons via DIET that mediated by ABC, and thus favorable for its enrichment as well as methanogenic improvement during sludge anaerobic digestion. Therefore, it was reasonable to conclude that ABC addition at the lower inoculation ratios were benefit for the enrichment of specific methanogens like *Methanosarcina*, and DIET that involving *Methanoscrina* species might be a primary mechanism for the methanogenic enhancement during sludge anaerobic digestion.

4. Conclusion

ABC addition enhanced methanogenesis at the lower inoculation ratios (4% and 1%, v/v) but not at the higher ratio (10%, v/v). Methanogenic improvements by ABC were not due to initial OLR increments at the lower inoculation ratios. Otherwise, ABC addition at the lower inoculation ratios were more favorable for *Methanoscrina* enrichment than the higher ratio, which might be benefit for methanogenesis through DIET. ABC addition enhanced methanogenesis by enriching specific methanogens (e.g. *Methanosarcina*) under low inoculation ratio during sludge anaerobic digestion. This finding would be valuable for waste reduction and energy recovery through anaerobic digestion enhancement.

CRediT authorship contribution statement

Qian Jiang: Methodology, Data curation, Writing - original draft, Investigation. **Chao Zhang:** Methodology, Formal analysis. **Ping Wu:** Writing - review & editing. **Peng Ding:** Writing - review & editing. **Yan Zhang:** Supervision, Writing - review & editing. **Min-hua Cui:** Supervision, Writing - review & editing. **He Liu:** Funding acquisition, Conceptualization, 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.

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Appendix A. Supplementary data

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