Simultaneous intensification of direct acetate cleavage and CO₂ reduction to generate methane by bioaugmentation and increased electron transfer

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HIGHLIGHTS
- Detailed analysis of multiple methane production pathways was conducted.
- Maximum H₂ production was promoted by ~3 and 4 times with bioaugmentation and CMs.
- Acetoclastic methanogenesis was beneficial from both bioaugmentation and CMs.
- Various CH₄ generation pathways were stimulated by CMs except for DIET-dependent CO₂.

GRAPHICAL ABSTRACT

ABSTRACT
Direct interspecies electron transfer coupled to CO₂ reduction, DIET-CO₂ reduction, to generate methane is proposed and prosperous in 2010s. It is well known that bioaugmentation and increased electron transfer benefit DIET-CO₂ reduction. Herein, we studied whether other methanogenic pathways, such as H₂-mediated methanogenic progress and direct acetate cleavage (acetoclastic methanogenesis), are simultaneously favorable in the presence of conductive materials (CMs). If so, contribution of DIET-CO₂ reduction may be overestimated because overwhelming studies just considered this pathway. Detailed researches on whether and how Clostridium pasteurianum coupled with CMs, granular activated carbon, biochar, nano-magnetite and graphene, influenced methanogenic progresses were conducted. Overall, C. pasteurianum enhanced methane production rate, which was further improved by some CMs. Combined with metabolism, kinetic and electrochemical analysis, experimental results showed that hydrogenotrophic methanogenesis occurred and bioaugmentation strengthened this progress, which was further motivated by CMs, such as biochar and magnetite. 16S rRNA gene analysis suggested that Methanobacteriaceae was potentially responsible for methane production. Whereafter, DIET-CO₂ reduction may become prosperous according to electrochemical and thermomechanical analysis. Acetoclastic methanogenesis was also triggered by bioaugmentation based on experiments by using inhibitor, CH₃F, for acetoclastic methanogenesis and carbon isotope fractionation. More importantly, magnetite and graphene, which significantly accelerated electron transfer based on electrochemical analysis, further stimulated acetoclastic methanogenesis. Methanotrix dominated and may play an important role in this stage. This work suggests that diverse methanogenic pathways may be benefited from an increase of electron transfer other than...
DIET-CO$_2$ reduction. Consequently, the long-standing view that only DIET-CO$_2$ reduction was stimulated by conductive materials may need to be reevaluated. Our research provides potential guides to increase methane production during anaerobic digestion by the enhancement of acetoclastic methanogenesis.

1. Introduction

Methane is actively involved in global carbon and energy cycles as a well-known greenhouse gas and renewable energy. Hydrolysis, fermentation, acetogenesis, and methanogenesis mainly consist of the whole process from macromolecular organic substances to methane production [1]. In the progress of fermentation, which may also be artificially intervened, acidogenic fermentation bacteria convert soluble monomers into terminal products, such as volatile fatty acid [2,3]. The formation of methane has traditionally been identified as a result of methanogen metabolism in the final step of organic matter fermentation in anaerobic terrestrial environments and artificial digestion [4]. Methanogenic progress typically occurs together with the activity of bacterial and fungal partners that form fermentation end products such as hydrogen, CO$_2$ and acetate as by-products of organic matter breakdown. In natural environments, CO$_2$ reduction contribute to ~1/3 of the total methane production, and the rest is mainly from acetoclastic methanogenesis [5]. Obviously, acetate cleavage serves as a primary way in methane production in terrestrial ecosystems, such as soils and sediments. In contrast, the artificial system is more concerned with the reduction of CO$_2$ [6,7].

A strong point of view always has been believing that reducing CO$_2$ to produce methane with hydrogen or formate as a shuttle ever since the first major breakthrough in the field came out half a century ago [8]. Early in the 2010s, as an alternative of interspecies H$_2$ transfer, direct interspecies electron transfer (DIET) was proposed and firstly found through cocultures of two species of *Geobacter* [9]. Inspired by this model, Lovley team further discovered possibility for DIET in methanogenic wastewater digester aggregates [10]. The use of DIET for methanogenesis between *Geobacter* species and methanogens was also confirmed in aggregates [11,12]. Simultaneous studies demonstrated that conductive materials (CMs), such as granular activated carbon (GAC) and magnetite (MT), stimulated syntrophic methane production between electron-donating microbes and methanogenic archaea [13,14]. Subsequently, a bulk of research articles published in the last 7 years on electromethanogenesis with the participation of CMs [15–18]. In light of these discoveries, increased electron transfer coupled with the reduction of CO$_2$ are considered to contribute to methane production within complex microbial communities. However, a notable study, which demonstrated that carbon nanotubes (CNTs) can promote methane generation with independence of the syntrophy between bacteria and archaea [19], reminds us to pay attention the long-held view that the stimulation of CMs can promote methane production via the necessary cooperation, i. e. DIET-CO$_2$ reduction. Furthermore, our recent findings suggested that enhanced electron transfer by carbon cloth may be favorable to acetoclastic methanogenesis [20]. Therefore, the favorable methanogenic pathways should be comprehensively analyzed in the presence of conductive materials/mineral.

In the process of anaerobic syntrophy, by utilization of fermentation productions, methanogenic archaea maintain a low level partial pressure of C1 and C2 to foster an energetically favorable condition for their syntrophic partners, which can persistently degrade compounds that are otherwise difficult to metabolize accompanied by energy harvesting [8]. As a beneficiary, methanogens make full use of the metabolic capacity of their cooperators to obtain more carbon source. In practice, the large amount of volatile fatty acids present in anaerobic sludge [21,22], and these substances cannot be directly utilized by methanogenic archaea except for acetate. Furthermore, it is widely accepted that primary sludge is easy to digest while secondary sludge or waste activated sludge are not. Although total COD of sludge is normally high, soluble COD of it which can be utilized to proceed acidogenesis is low [1]. Therefore, by increasing the activity and/or abundance of syntrophic partners, it is theoretically possible to increase the supply of methanogenic substrates to further strengthen sewage treatment. However, study on bioaugmentation coupled with application of CMs is very rare.

Recent anaerobic digestion (AD) studies tried to exploit an enhancement of electron exchange associated with DIET for the treatment of organic waste streams and have reported improved methane production rates when promoting DIET-CO$_2$. While DIET alone does not explain enhanced AD performance in many cases [23]. In this study, we attempted to comprehensively analyze the response of methanogenic pathways to bioaugmentation and CMs. The dominant hydrogen producing microorganisms used in dark fermentative biobiohydrogen are affiliated to *Clostridium* [24]. Very recently, we conducted study on biohydrogen using *Clostridium pasteurianum* with the ability to ferment glucose to acetate and H$_2$ [25], which are very important substrates for methanogenic progress. Therefore, this kind of bacteria was used to strengthen production of methanogenic substrates. In order to make the research conclusion more universal, four kinds of CMs were evaluated, including GAC, biochar, magnetite and graphene. Combining stable isotope fractionation, thermomechanical calculation, electrochemical analysis, whether and how bioaugmentation, together with CMs, affects the carbon and/or electron-flow pathways involved in the fermentative and methanogenic processes were explored.

2. Materials and methods

2.1. Batch experiments

Anaerobic granular sludge (AGS) was collected from a full-scale upflow anaerobic sludge bed (UASB) reactor treating beer production wastewater (Beijing, China). The collected sludge was stored in the refrigerator at 4 °C for use. Prior to the experiments, the sludge was acclimated to a simple medium in an anaerobic bottle (2 L) for more than three months [26]. The composition of the medium was as follows (L): glucose 3.6 g, NaHCO$_3$ 3.33 g, NH$_4$Cl 0.84 g, KH$_2$PO$_4$ 0.085 g. *C. pasteurianum* DSM525 was purchased from the DSMZ and cultured in the mineral salt glucose (MSG) medium. The MSG recipe can be referred to Zhang et al. [25]. It is briefly described as follows (L): peptone, 0.5 g; tryptone, 0.5 g; cysteine hydrochloride, 0.5 g; glucose, 10 g; NaCl, 5 g; KH$_2$PO$_4$, 0.54 g; K$_2$HPO$_4$, 2.10 g; vitamin solution, 10 mL; and mineral elements solution, 10 mL. The pH was adjusted to 6.5 ± 0.1.

AGS (5 mL) was dispensed into sterile 12-mL serum vials, which were pre-evacuated, flushed with high-purity nitrogen gas. In order to prepare seed solution of *C. pasteurianum*, 500 mL MSG medium was prepared with 5% inoculum. Then, it was cultured at 30 °C in the dark without shaking for 24 h. The culture (500 mL) containing *C. pasteurianum* was concentrated by centrifugation (6000 rpm, 5 min, 4 °C) and resuspended in 5 mL sterile water named as suspension liquid. For bioaugmentation by *C. pasteurianum*, each vial was received 0.1 mL suspension liquid. The dosage of four kinds of CMs used were listed in Table 1. For the dose used, our principle is to try to select the intermediate amount or frequently used amount in previous studies [15,27]. The details of materials/mineral can refer to previous studies [13,16,18,28]. In brief, for the GAC used in this study, it is the same as the GAC (Sigma-Aldrich, St Louis, MO, USA) used in a previous research with particle size about 8–20 mesh [13]. The conductivity of GAC measured with the two-electrode system was about 3 mS/cm. The
biochar used in this study was made in a vertical kiln made of refractory bricks in Sanli New Energy Company, Henan, China. It was produced from wheat straw by pyrolysis at 350–550 °C. The conductivity of biochar was ~14.3 S/cm. The nano-Fe3O4 particles were synthesized by using a conventional aqueous co-precipitation method. In brief, FeCl2 and FeCl3 were dissolved in deoxygenated HCl solution. Then NaOH solution was added drop-wise under vigorous stirring. Fe3O4 particles were subsequently generated with characterization of obvious black precipitate. The particles were purified by centrifugation and suspension in deoxygenated water. It was washed repeatedly with deoxygenated water until the wash water was neutral. Finally, the precipitate were collected by using a magnet. The size of most particles was in the range of 20–100 nm. Graphene was purchased from Nanjing XF NANO Materials S&T Co., Ltd. China detailed in previous study [28]. In brief, the parameters are as follow: diameter, 0.5–2 μm; thickness, 0.8–1.2 nm; single layer ratio, 80%. The control is the treatment without the addition of C. pasteurianum and CMs. Extra three cycles of vacuum/charging nitrogen gas were applied to keep vials in a strictly anaerobic state. All experiments were carried out under static conditions of 30 °C in the dark.

### 2.2. Metabolism analysis

Vials were sacrificed in triplicate to determine methane, CO2 and hydrogen concentration after a certain incubation period (4, 8, 12, 20, 30, 40, 50, 63 and 73 h). This determination was to analyze methane production potential and the possible methane production pathways. The gases were tested using a gas chromatograph (GC; Agilent 7820A, USA) equipped with a flame ionization detector for methane and CO2 and thermal conductivity detector for hydrogen. High-pressure liquid chromatography (HPLC; Agilent 1260 Infinity) was used to test acetate, butyrate and glucose concentration. The detailed operation can refer to recent report [25]. In brief, high-pressure liquid chromatography (HPLC; Agilent 1260 Infinity) was used. Separation was fulfilled using a Hi-Plex H column at 60 °C with a refractive index detector at 55 °C. The mobile phase used was 5 mM H2SO4 at a flow rate of 0.6 mL min⁻¹. The minimum detectable limit for acetate was approximately 0.02 mM. CH3F (-1.5%) was used to inhibit acetoclastic methanogenesis [5,29]. If the consumption of acetate is mainly to produce methane by direct acetate cleavage, significant acetate accumulation occurs when the CH3F is added. Simultaneously, the production of methane will be retracted.

### 2.3. Kinetic and thermomechanical analysis

Referring to previous report [30], two models were used to analyze the scenario of methane production to evaluate the function of bioaugmentation and CMs. The formulas for the two models are as follows:

**FitzHugh model:**

\[
P(\lambda) = \frac{1}{k} \ln \left(1 + e^{-k(t-\lambda)}\right)
\]

**Transference model:**

\[
P(\lambda) = \frac{1}{k} \ln \left[1 - \exp \left(-R_{\text{max}} \times (t-\lambda)/P_{\text{max}}\right)\right]
\]

where \(P\) is the cumulative methane concentration (mmol L⁻¹) at time \(t\), \(P_{\text{max}}\) is the maximum methane concentration at the end of incubation (mmol L⁻¹), \(t\) is time (h), \(k\) is the first-order reduction rate constant for the methane production (h⁻¹), \(R_{\text{max}}\) is the maximum methane production rate (mmol L⁻¹ h⁻¹), \(\lambda\) is the lag period (h), and \(e\) is 2.71828.

Concentrations of gases and acetate were used to calculate the ΔΔG of hydrogenotrophic methanogenesis and acetoclastic methanogenesis to evaluate the possible contribution on methane accumulation. In brief, ΔΔG of hydrogenotrophic methanogenesis can be calculated as follow,

\[
\Delta G = \Delta G^0 + RT \times \ln \left(\frac{C_{\text{CH}_4}}{C_{\text{CO}_2} + C_{\text{H}_2}}\right)
\]

\[
\Delta G^0 = \Delta G \text{ at 273.15 K and 101.325 kPa}; R, \text{ the ideal gas constant, 8.3145 J mol}^{-1} K^{-1}; T, \text{ the absolute thermodynamic temperature, 303.15 K}; C_{\text{CH}_4}, C_{\text{CO}_2}, \text{ and } C_{\text{H}_2}\text{ are concentrations of methane, CO2, and H2, respectively, mol L}^{-1}. \text{The calculation of } \Delta G^0\text{ was detailed in our previous report [16]. For acetoclastic methanogenesis,}
\]

\[
\Delta G = \Delta G^0 + RT \times \ln \left(\frac{C_{\text{CH}_4} + C_{\text{CO}_2}}{C_{\text{acetate}}}\right) + 2.303 \times RT \times N_{\text{tot}} \text{ holds,}
\]

where the parameters are the same as above. \(C_{\text{acetate}}\) is concentration of acetate, mol L⁻¹; \(N_{\text{tot}}\) is pH value.

### 2.4. Electrochemical analysis

Microbial fuel cell (MFC) was employed to analyze the electron transfer efficiency to study whether, or not, bioaugmentation and CMs can accelerate electron output and transfer. H-type double chamber MFCs were used and the chambers were separated by a cation exchange membrane (Ultrx CM-7000). Graphite plate (3.0 cm × 2.5 cm × 0.3 cm) was used as the electrode in each chamber and connected with titanium wire. A resistor (1 kΩ) was equipped in the external circuit. AGS (80 mL) was added in the anode chamber and an identical quantity of K3[Fe(CN)6] (0.05 M) was added to the cathode chamber. Nitrogen flushing (20 min) was conducted in the two chambers to eliminate air. A data acquisition system (Model 2700, Keithley Instruments, USA) and ExcellLINX software were utilized to acquire and record the output voltage. The current density, \(C_{\text{den}}\) (A/m²) was calculated using the following expression:

\[
C_{\text{den}} = \frac{U}{S}\text{, where } U \text{ is the output voltage (V), } S \text{ is the surface area of the electrodes (m²).}
\]

### 2.5. Carbon isotope fractionation and calculation

Carbon stable isotope fractionation and calculation were conducted to analyze methanogenic progress. Methane and CO2 collected from the headspace were tested for obtaining the δ13C using a gas chromatograph combustion isotope ratio mass spectrometer (GC-C-IRMS) system (Thermo Fisher MAT253, Germany). The isotopes were quantified in a Finnigan MAT253 IRMS. Separation of CH4/CO2 was performed in a Finnigan Precon. Gas (~1 mL) was injected into a sample container (100 mL), which was filled with helium gas (99.999% purity) beforehand. A chemical trap (filled with Mg(ClO4)2 and ascarite) was applied to scrub CO2 from the sample. Methane was subsequently purified by cold trap with the help of Ni wires. Then, methane can be oxidized in a combustion reactor at 960 °C and converted to CO2 and water. The combusted CO2 was enriched by two liquid nitrogen cold traps and transferred into the IRMS for determination. The precision of repeated analyses was ± 0.2% when 1.3 mmol methane was injected. The abundance of 13C in a sample is given relative to a standard using the δ notation:

\[
\delta^{13}C = \left[\frac{\text{ratio}_{\text{sample}}}{\text{ratio}_{\text{PDB}}} - 1\right] \times 1000,
\]

where PDB refers to the Pee Dee Belemnite carbonate that is used as standard which has a 13C/12C ratio of 0.0112372. A similar method was
used to test $\delta^{13}$C-values of CO$_2$. The chemical trap was replaced by a water trap. The $\alpha$ value can be calculated using the equation:

$$\alpha = \frac{\delta^{13}\text{CO}_2 + 1000}{\delta^{13}\text{CH}_4 + 1000}.$$  

2.6. Determination of archaea diversity

Samples collected at the 50th hours was used to analyze the potential contributors on methane production. Polymerase chain reaction (PCR) amplification of 16S rRNA gene was performed using archaeal primers (Ar515f and Ar907r) [31]. An amplicon library was built followed by sequencing using an Illumina Miseq platform from Tiny Gene Bio-Tech (Shanghai) Co., Ltd. For analysis of bioinformation, the raw reads, low-quality sequences, clustering operational taxonomic units (OTUs), and taxonomic classifications were processed [16].

2.7. Statistical analysis

Data are presented as mean ± standard deviation of triplicate cultures expect for carbon isotope fractionation and calculation, in which two repetitions was conducted. All statistical analyses were performed with SPSS 19.0 (SPSS Inc., Chicago, USA) and Origin 8.5 (Origin Lab Corporation, USA) software. A one-way analysis of variance with HSD’s test was used to analyze the significance level, and a P value < 0.05 was considered statistically significant.

3. Results and discussion

3.1. Effects of C. pasteurianum and CMs on methane production

In this part, we tried to analyze whether C. pasteurianum and CMs can enhance methane production. Obviously, C. pasteurianum stimulated methane production (Fig. 1). Transference and Fitzhugh models were used to fit actual measured values to obtain methane production potential (MPP) (Tables 2 and 3). The results showed that the MPP in the control group were 14.11 ± 0.63 and 14.5 ± 0.89 mM according to Transference and Fitzhugh models, respectively. The calculation results by the two models were almost identical, which proved the correctness of the model selection. After the addition of C. pasteurianum, the MPP presented by Transference and Fitzhugh models were 25.3 ± 2.7 and 25.14 ± 2.41 mM, which were increased by 79.31% and 73.43% compared to the control. Therefore, bioaugmentation seemed to be a robust strategy to excavate MPP. Based on the syntrophy between fermentative bacteria and methanogens, it is reasonable that the fermentation progress was also improved. One study also found that the increased percentage of order Clostridiales, which can degrade carbohydrate, sugar, amino acid, etc., caused the raise of acetate and methane production [32]. A recent study accelerated methane production via adding Geobacter sulfurreducens PCA in anaerobic digestion system [33]. The maximum methane production rate was increased from 0.09 mmol/d in control to 0.16 mmol/d in experimental groups, increased by 77.78%. As a matter of experience, they suggested that DIET-CO$_2$ reduction to generate methane was enhanced [33].

A plethora of literature provided evidences that DIET-dependent methane production by reducing CO$_2$ contributed to anaerobic sludge treatment with the help of CMs [15,34,35]. However, it was found that acetate production in the fermentative step is the main driver of the overall methane production [36]. Therefore, in this study, we want to comprehensively explore if CMs can bring another value-added benefit, such as increased direct acetate cleavage, other than increased DIET-CO$_2$ reduction companied by the reinforcement of fermentative step by bioaugmentation. Easy-to-access and non-precious non-nanomaterials were the primary research object, such as GAC and biochar. Also, in the seven-year period of 2012–2018, it can be observed that studies about the acceleration of methane production by nanomaterials have been in full swing [15]. In order to expand these research findings, whether or not nanoparticles (magnetite) and nano-overlayers (graphene) can work was explored as well.

According to the Transference model, the $R_{\text{max}}$ were enlarged by all materials indicating that the performance of methane production was
more favorable with the participation of CMs. These findings were more consistent with previous reports [13,15,17]. In contrast to non-nanomaterials, nanomaterials showed better performance (Table 2). In addition to $R_{\text{max}}$, $P_{\text{max}}$ is also a very critical indicator for methane generation. However, on the basis of bioaugmentation, CMs did not bring additional benefits to $P_{\text{max}}$. Increasing the methane yield by bioaugmentation may be more effective, and the application of CMs is best aimed at increasing the rate of methane production.

Table 2
Fitting parameters of transference model.

| Treatment            | Kinetic model parameters | $R_{\text{max}}$ | $P_{\text{max}}$ | $R^2$ |
|----------------------|--------------------------|------------------|------------------|------|
| Control              | $1.678 \pm 0.847$        | 0.756 $\pm 0.092$ | 14.108 $\pm 0.633$ | 0.999 |
| C. pasteurianum      | $-0.161 \pm 1.432$       | 0.631 $\pm 0.084$ | 25.297 $\pm 2.697$ | 0.999 |
| C. pasteurianum + GAC | $0.533 \pm 1.297$       | 0.811 $\pm 0.118$ | 19.959 $\pm 1.339$ | 0.999 |
| C. pasteurianum + Biochar | $0.403 \pm 1.1$       | 0.811 $\pm 0.094$ | 22.986 $\pm 1.418$ | 0.999 |
| C. pasteurianum + MT  | $1.107 \pm 1.23$        | 0.916 $\pm 0.13$  | 24.346 $\pm 1.746$ | 0.998 |
| C. pasteurianum + Graphene | $0.073 \pm 1.074$    | 0.865 $\pm 0.95$  | 24.1 $\pm 1.358$  | 0.999 |

Table 3
Fitting parameters of Fitzhugh model.

| Treatment              | Kinetic model parameters | $P_{\text{max}}$ | $R^2$ |
|------------------------|--------------------------|------------------|------|
| Control                | $14.498 \pm 0.888$       | 0.999            |
| C. pasteurianum        | $25.144 \pm 2.407$       | 0.999            |
| C. pasteurianum + GAC  | $20.191 \pm 1.333$       | 0.999            |
| C. pasteurianum + Biochar | $23.224 \pm 1.372$     | 0.999            |
| C. pasteurianum + MT   | $24.967 \pm 1.911$       | 0.998            |
| C. pasteurianum + Graphene | $24.14 \pm 1.398$    | 0.999            |

3.2. Bioaugmentation and CMs stimulated hydrogen accumulation

Since bioaugmentation and CMs can accelerate the production of methane, which pathways strengthen it? We separately analyzed the feasibility of hydrogenotrophic methanogenesis, DIET-CO$_2$ reduction and acetoclastic methanogenesis. Experimental results demonstrated that hydrogen concentration markedly changed after the addition of C. pasteurianum (Fig. 2a). The accumulation of hydrogen occurred during the first 8 h, suggesting that the amount of hydrogen produced was higher than its consumption. The peak of hydrogen concentration in control group was $\sim 0.1$ mM, and it reached the highest point with value about 0.3 mM by bioaugmentation. Obviously, the accumulation of hydrogen was tripped by C. pasteurianum. The rate of methane production was also enhanced (Fig. S2). Moreover, the addition of materials seemed to further enhance the production of hydrogen, suggesting that CMs, such as biochar and magnetite, may be beneficial to C. pasteurianum and/or indigenous microorganisms with fermentation function in anaerobic sludge [37,38]. In fact, compared to treatments of the control and bioaugmentation groups, methane production rate were higher in the presence of CMs. These results suggested that hydrogenotrophic methanogenesis may contributed methane production. Moreover, bioaugmentation and CMs enlarged methane yield by this pathway.

Fig. 2. The effects of non-nanomaterials on dynamics of hydrogen concentration without (a) and with CH$_3$F (c); Changes of hydrogen concentration with the addition of nanomaterials in the absence (b) and presence (d) of CH$_3$F.
It should be noted that the hydrogen concentration was one order of magnitude lower than produced methane. Even if instantaneous generation and consumption of hydrogen occurred, hydrogenotrophic methanogenesis was only apparent in the early stage from the thermodynamic point of view. The results showed that when the experiment progressed to about the 40th hour, the $\Delta G$ of hydrogenotrophic methanogenic process approached zero (Fig. 3a and b). After that, the methane production process with hydrogen as the reducing power was disadvantageous.

In the second half of the experiment, it was impressive that the control treatment reflected a very weak ability to continuously produce methane. In contrast, it still showed the continuous accumulation trend of methane for others treatments (Fig. 1a and b). Taking the group with bioaugmentation as an example, the yield of methane produced in this stage accounted for 35.43% of the total. Therefore, other way(s) contributed significantly to methane production except for potential hydrogenotrophic methanogenesis, such as the acetoclastic methanogenic pathway and DIET-CO$_2$ reduction.

3.3. Potential DIET-CO$_2$ reduction contributed to methane production

According to the fitted data by Transference model, GAC reduced $P_{\text{max}}$. Therefore, the electrochemical and carbon isotope analysis of this treatment was not further determined. The control group showed very low electrical activity with the current density within the range of $\sim 0.01$ and 0.02 A/m$^2$ (Fig. 4a). From the change of current density with the addition of $C. pasteurianum$, it was greatly improved. Therefore, the increase in methane concentration may be related to the greater supply of reducing power with electron as the more direct form. Ren et al. revealed that $Ruminiclostridium$ $cellulolyticum$ (formerly known as $Clostridium$ $cellulolyticum$) fermented cellulose, and these fermentation products fuelled current production by $G. sulfurreducens$ [39]. After 30 h, biochar markedly accelerated electron transfer. Accordingly, the amount of methane produced was higher than that of the bioaugmentation group under both two treatments with or without inhibitor (Fig. 1a and c). Therefore, potential DIET-CO$_2$ reduction may contribute to methane production in the middle and late stages.

For the treatments of adding nanomaterials, current density was greatly improved. The peak current values of both treatments with magnetite and graphene increased to 2.5 times of the experiment with the $C. pasteurianum$ only. Correspondingly, more electric quantity can be obtained (Fig. 4b). Obviously, magnetite and graphene triggered higher $R_{\text{max}}$ synchronously with high current density. Overall, for the reduction of CO$_2$ to produce methane, the reducing power was more likely in the form of hydrogen in the early stage, and DIET-CO$_2$ reduction may had a contribution in the middle and later stages. Study from Storck et al. showed that external limitations can be compensated for by the increase of metabolic efficiency when using DIET compared to mediated interspecies electron transfer via hydrogen [40]. The phenomenon observed in this study may be due to the reason that DIET can provide more energy for microorganisms than using indirect electron transfer where hydrogen molecule acts as a shuttle for electron transfer [7]. Furthermore, except for direct electron transfer mediated by some materials, the synthetic materials, such as $\text{Ag}_3\text{PO}_4@\text{MWCNTs}@\text{PANI}$, and $\text{Ag}_3\text{PO}_4@\text{MWCNTs}@\text{Cr:SrTiO}_3$, play an role in stimulating production of photogenerated electrons [41,42]. Application of these materials seems to be favorable to an increase of methane production in future study and application.

3.4. Bioaugmentation stimulated acetoclastic methanogenesis

Under the premise of adding CH$_3$F to inhibit methane generation by direct acetate cleavage, acetate concentration decreased very slowly (Fig. 5c and d) and even a considerable amount of acetate accumulated (> 3 mM) was observed. Accordingly, methane production was negatively affected (Fig. 1c and d). A manifest state of acetate consumption was presented accompanied by obvious methane accumulation in the absence of CH$_3$F (Fig. 5a and b). It can be concluded that the production of methane was extremely dependent on the acetoclastic methanogenesis pathway. Only a weak accumulation of methane in the control group at the later stage suggested that all methanogenic pathways almost no longer worked (Fig. 1a). In contrast, there was obvious
Fig. 4. The change in current density (a) and total electric quantity (b) in the MFCs over time. The potential methanogenic pathways (c) and the calculated $\alpha$ values according to $^{13}$C of methane and CO$_2$ (d). Background values referred to a previous report [47].

Fig. 5. The effects of non-nanomaterials on dynamics of acetate concentration without (a) and with CH$_3$F (c); The effects of nanomaterials on dynamics of acetate concentration without (b) and with CH$_3$F (d).
methane production due to the bioaugmentation. Specially, only trace methane accumulation can be detected with the addition of inhibitor in the bioaugmentation treatment (Fig. 1c). These results provide reliable evidence that bioaugmentation triggered more methane production by direct acetate disproportionation.

We also analyzed the ΔG of the acetoclastic methanogenic pathway (Fig. 3c and d). It was energy favorable during all experimental process. From this perspective, acetoclastic methanogenesis pathway can continuously occur. In order to confirm the possibility, stable isotope fractionation experiments were performed to figure out methane source. Bioaugmentation by C. pasteurianum did not influence ΔG values within the range from 1.03 to 1.035. According to previous reports, this data is clearly in the numerical range of acetoclastic methanogenic pathway [5,43]. It should be emphasized that although we try to remove the CO2 doped in methane with chemical trap, incomplete removal of CO2 may make the value of ΔG smaller leading to an overestimation of the contribution of acetate cleavage.

Because of the instantaneous production and consumption of methanogenic substrates, such as acetate, we would like to know more about whether some volatile fatty acids played a role in this process. The data of butyrate concentration provided a gradually decreasing scene, and it approached complete consumption at the late stage (Fig. S3). Intriguing, suppression of acetoclastic methanogenesis can retrait the rate of butyrate consumption. These results suggested that the decomposition of butyrate into acetate was also beneficial to the methane production. Apart from this, bioaugmentation triggered more butyrate production, which acted as an indirect substrate for methanogens. This may also be one of the reasons for increasing MMP by adding C. pasteurianum.

### 3.5. Acetoclastic methanogenesis further benefited from the addition of CMs

For the impact by accelerated electron transfer, almost all that is known about the benefits are to reduce CO2. Not disregarding that the function of increased DIET-CO2 reduction introduced by CMs, it hardly explain all the reported observations [23]. In fact, DIET-CO2 reduction to produce methane was only unequivocally confirmed in cocultures of Geobacter with methanogens [11,12]. Anyway, many studies showed that the microbial community without traditional electrogenic bacteria still advocated this theory. Logan et al. concluded that providing more attachment sites by conductive materials can accelerate the growth of versatile methanogens [44], which may stimulate the direct acetate cleavage to produce more methane. Our recent study on soil samples showed that, in comparison to cotton cloth, conductive carbon cloth showed potential impact on the acetoclastic methanogenic pathway by electrochemical analysis and carbon stable isotope analysis [20]. This result suggested that, in addition to providing attachment sites, the conductive properties may also function on acetoclastic methanogenesis.

Most studies mentioned that the confirmation of methanogenic process was done by electrochemical analysis and sequencing analysis in the anaerobic sludge. The reliability of these approaches may be insufficient compared to isotope analysis. In this study, the addition of CMs significantly reduced values of α compared to only bioaugmentation (Fig. 4b and c), suggesting that CMs further enhanced acetoclastic methanogenesis pathway. It was reported that magnetite also facilitated consumption of H2 by functional microbes to produce more acetate [45]. In this case, the contribution of CO2 reduction may be further weakened and direct acetate cleavage will be strengthened. In addition, based on metabolic analysis, CMs did not promote or even reduce the rate of methane production in the absence of acetoclastic methanogenesis (Fig. S2c and d). In sharp contrast, these materials did enhance methane production rate when direct acetate cleavage was involved (Fig. S2a and b). That is, the enhancement of methane production rate was very likely to come from the contribution of acetoclastic methanogenesis. Unlike most studies that focus on CO2 reduction process [6,7,15,16,46], this study raises a hint that the promotion of methane production by CMs may not be completely through this pathway.

#### 3.6. Methanothrix and Methanobacteriaceae potentially contributed to methane production

The genus level identification of archaea community structure of the five treatments (Table 1 except for C. pasteurianum + GAC) is illustrated in Fig. 6. The dominant genus of archaea was Methanothrix (formaly Methanosacetaceae), which can use both acetoclastic methanogenesis and DIET-CO2 reduction to generate methane [11]. It was enriched in the treatments with the addition of C. pasteurianum and CMs. Methanothrix may mainly contribute to methane production in this study and the stimulation of methane production rate seemed to be related to the increase in its abundance. Furthermore, the abundance of Methanobacteriaceae was higher in the treatment with bioaugmentation compared to the control treatment. Particularly, all treatments for adding CMs further enhanced Methanobacteriaceae abundance. Methanobacteriaceae is the canonical hydrogenotrophic methanogens [19]. The contribution of the hydrogenotrophic methanogenesis on methane production was in good agreement with the abundance change of Methanobacteriaceae. That is, both Methanobacteriaceae abundance and methane yield form hydrogenotrophic methanogenesis followed this pattern: control treatment < bioaugmentation < application of CMs. Therefore, this kind of methanogen seemed to play a key role in methane production at the early stage.

### 4. Conclusions

Three methane production pathways contributed to methane generation in this study. Hydrogenotrophic methanogenesis actively participated in the early stage. Bioaugmentation accelerated this progress. Intriguingly, reduction CO2 by hydrogen can be further promoted in the presence of both non-nanomaterials and nanomaterials. In the middle of the experiment, the reducing power may be more directly involved in the reduction of CO2 in the form of electrons rather than hydrogen. Similarly, methane generation was stimulated by both bioaugmentation and CMs, which can triggered much faster of electron transfer. As the experiment progressed, evidences from both carbon stable isotope fractionation and inhibition experiment suggested that acetoclastic
methanogenesis was also strengthened by CMs. To the best of our knowledge, this is the first discovery that other methanogenic pathway(s), except for DIET-CO₂ reduction, may also benefit from the increase of electron transfer rate in process of anaerobic digestion of organic wastes.

Author contributions

L.X. and F.L designed the research. L.X., R.S., P.Z., Y.T., L.J. and Z.Y. performed experiments. L.X., Z.S. and F.L analyzed the data. L.X. and P.Z. wrote the article.

Declaration of Competing Interest

The authors declare that they have no competing financial interest associated with this work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cej.2019.122229.

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