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Authors	Wu, Benteng;Lin, Richen;Kang, Xihui;Deng, Chen;Xia, Ao;Dobson, Alan D. W.;Murphy, Jerry D.
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Article

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Graphene addition to digestion of thin stillage can alleviate acidic shock and improve biomethane production

Benteng Wu [1, 2], Richen Lin [1, 2, *], Xihui Kang [1, 2, 3], Chen Deng [1, 2], Ao Xia [4], Alan D.W. Dobson [5], Jerry D. Murphy [1, 2]

[1] MaREI Centre, Environmental Research Institute, University College Cork, Cork T23 XE10, Ireland

[2] School of Engineering, University College Cork, Cork T12 YN60, Ireland

[3] Guangzhou Institute of Energy Conversion, Chinese Academy of Sciences, Guangzhou 510640, China

[4] Key Laboratory of Low-grade Energy Utilization Technologies and Systems, Chongqing University, Ministry of Education, Chongqing 400044, China

[5] School of Microbiology, University College Cork, Cork T12 K8AF, Ireland

[*] Corresponding author: Dr. Richen Lin, MaREI Centre, Environmental Research Institute, University College Cork, Ireland. Tel.: +353 (0)21 490 1948. Email: richen.lin@ucc.ie

Abstract:

Production of biomethane from distillery by-products (such as stillage) in a circular economy system may facilitate a climate neutral alcohol industry. Anaerobic digestion (AD) of easily degradable substrates can lead to rapid acidification and accumulation of intermediate volatile fatty acids, reducing microbial activity and biomethane production.

Carbonaceous materials may function as an abiotic conductive conduit to stimulate microbial electron transfer and resist adverse impacts on AD. Herein, nanomaterial graphene and more cost-effective pyrochar were comparatively assessed in their ability to recover AD performance after acidic shock (pH 5.5). Results showed that graphene addition (1.0 g/L) could lead to a biomethane yield of 250 mL/g chemical oxygen demand; this is an 11.0% increase compared to that of the control. The recovered process was accompanied by faster propionate degradation ($CH_3CH_2COO^- + 2H_2O \rightarrow CH_3COO^- + CO_2 + 6H^+ + 6e^-$). The enhanced performance was possibly ascribed to the high electrical conductivity of graphene. In comparison, pyrochar addition (1.0 and 10.0 g/L) did not enhance biomethane yield, though it reduced digestion lag-phase time by 18.1% and 12.2% compared to the control, respectively.

Microbial taxonomy analysis suggested that *Methanosarcina* (81.5% in abundance) with diverse metabolic pathways and OTU in the order *DTU014* (6.4% in abundance) might participate in direct interspecies electron transfer contributing to an effective recovery from acidic shock.

Keywords: Anaerobic digestion; Biomethane; Conductive materials; Acidic shock; Thin stillage

Introduction

The transport sector is one of the largest and fastest increasing energy consumers. It is also the most challenging to produce climate friendly fuels for trucks, buses, ferries and planes; this sector is not readily suitable for electrification. The European Union recast Renewable Energy Directive (2018/2001) requires that the contribution of renewable energy in the transport sector is at least 14% by 2030, and that for advanced biofuels the contribution should reach 3.5%.[1] Advanced biofuels refer to fuels that do not require arable land for cultivation or use feedstocks that could be food. Typical feedstocks for advanced biofuels include animal manure, algae, crop residues and municipal solid waste.[1] Anaerobic digestion (AD) is an effective bioconversion technology which produces biomethane from wet organic material.[2, 3] Integration of AD with a sustainable waste management system can offer negative emission transport fuel.[4]

Conversion of grain to ethanol is a significant economic asset in many countries.[5, 6] For instance, whiskey production in Ireland has increased by 131% on a volume basis in the past 10 years.[7] However, in a conventional ethanol production process, up to 20 L of stillage can be generated for every litre of ethanol, producing a considerable quantity of organic by-products.[8] After solid/liquid separation of stillage, the liquid fraction (thin stillage) generally contains high concentrations of carbohydrates, proteins and other fermentation by-products; stillage displays a high chemical oxygen demand (COD) and low pH (3.5-4.5).[6] Unlike the solid fraction, there is a significant energy input to produce wet distillers solubles (a source of animal fodders) through evaporation of water from stillage due to its high water content.[9] Considering its high biodegradability, thin stillage can be used to produce biogas, which can in turn be used to satisfy some of the thermal and electrical energy demands of the distillery; this improves the sustainability of alcohol production and reduces reliance on fossil-based energy.[7] A further use of the produced biogas is to upgrade to biomethane for use as a sustainable climate friendly transport fuel. Typically transport fuel has a higher exergy than heating and more revenues are available in substituting for transport fuel than for heat.

However, in practice, digestion of readily biodegradable feedstock can encounter instability, experience lower biomethane production, and sometimes even failure. These issues may be attributed

to the particularly lower pH within AD systems resulting from the accumulation of volatile fatty acids (VFAs), and inhibition of subsequent methanogenesis.[10] The inhibition on AD performance caused by VFAs is due to their acidity rather than direct toxicity.[11] VFAs are not in of themselves toxic. Generally, they are produced and consumed as food and nutrients by microbes in a well-operated digester. Their inhibitionary effects are indirect as they lower the pH to an undesirable level and subsequently inhibit methanogenisis. In this context, some strategies have been adopted to enhance the stability of AD systems.[8, 12] Maintenance of a suitable pH within digesters maybe employed through addition of alkaline chemicals.[12] However, once these chemicals are consumed, acidification may occur again. Serious events which result in acidic suppression of microorganisms in AD, require long periods of operation to recover.[13] To maintain the stability of AD systems treating readily degradable feedstock, especially after experiencing episodes of external stress, more sustainable and effective methods should be considered.

Recently, carbonaceous conductive materials such as pyrochar, carbon cloth and graphene have been reported as a means to enhance system stability and improve biomethane production efficiency.[14-16] Carbon cloth could enhance AD stability through mitigation of acidic inhibition (pH as low as 5.0) and accelerate the recovery of the methanogenesis function due to the promoted direct interspecies electron transfer (DIET) between microbes.[13] Similar positive effects were observed when using pyrochar and granular activated carbon (GAC) to alleviate ammonia (NH_4^+-N) inhibition.[17, 18] Florentino et al. found that under high ammonia concentration (2.8 g NH_4^+-N/L) the biomethane yield increased by up to 53.4% through the addition of GAC, which enhanced syntrophic metabolism by providing high electrical conductivity between microorganisms.[18] Carbon-based conductive materials have been shown to enhance the degradation of VFAs such as butyrate and propionate, in turn leading to a high methanogenesis efficiency.[19] In a typical syntrophic methanogenesis process, the reaction occurs close to thermodynamic equilibrium; as such a minor disturbance in intermediates or substrates can lead to a shift of the metabolic pathway.[20] Unlike utilizing hydrogen or formate as the electron carrier, non-biological conductive materials are able to serve as electron conduits to transfer electrons between bacteria and archaea without the requirement

of synthesizing electrically conductive pili (e-pili) or nanowires, which typically are the biological electron shuttles to facilitate DIET.[21] This unique cell-to-cell electron exchange metabolism offers advantages to methane production from specific VFAs (such as propionic acid and butyric acid), which are susceptible to interference from the traditional electron carrier H_2. The degradation and methanogenesis process for model substrates of carbohydrates, proteins and alcohols (glucose, glycine and ethanol respectively) were shown to be accelerated and stabilized due to the establishment of DIET.[16, 17, 22] Given the high content of carbohydrates, proteins and alcohols in thin stillage, it is postulated that conductive materials can stimulate DIET in digestion of thin stillage. It is therefore hypothesised that stimulating DIET via conductive materials can alleviate the acidification stress and accumulation of VFAs, thus facilitating the recovery from severe acidic shock.

The innovation of this study is that it is the first investigation of the potential role of carbonaceous materials in digestion of thin stillage with external stress (in this case acidic shock). The objective of this study is to investigate the application of nanomaterial graphene and cost-effective pyrochar in digestion to resist an acidic shock (pH 5.5). The mechanics of system recovery were evaluated in terms of process stability (as measured by VFAs accumulation and pH change), biomethane production and responses of microbial community. The thermodynamic advantage of DIET was exemplified using propionate (a typical VFA observed in AD) as a model substrate.

Materials and Methods

Inoculum, feedstock and material

The inoculum for AD start-up was sourced from a lab-scale mesophilic (37 °C) reactor and has digested a wide variety of feedstocks such as grass silage, cattle manure, seaweed, and food waste over the years. Thin stillage, taken from an Irish whiskey distillery (Ireland), was used as feedstock. The characteristics of the inoculum and thin stillage are shown in Table S1.

Graphene nanosheets (length less than 2 μm) were purchased from Sigma Aldrich and the properties were detailed in our previous study.[22] Pyrochar was obtained from a local pyrolysis plant (Premier Green Energy, Ireland) pyrolyzing wood waste at 700 °C. The carbon content of pyrochar

was 87.8% on a total solid (TS) basis. Other characteristics of pyrochar such as the pH value of suspension, scanning electron microscope images and X-ray diffraction pattern were shown in a previous study.[23] Pyrochar samples were ground and sieved to obtain a practical size less than 150 μm. Prior to AD, graphene and pyrochar were dried at 105 °C to ensure the complete removal of moisture content. To ensure a well-mixed graphene/pyrochar solution in the digestate/inoculum suspension, different dosages of conductive materials were mixed in distilled water and added in the glass fermenter, then the fermenter was shook vigorously in a horizontal direction until a homogeneous black solution was formed, as illustrated in Figure S1.

Experimental start-up

Batch AD experiments were conducted using an Automatic Methane Potential Test System (AMPTS II) (Bioprocess Control, Sweden).[22] In each individual digester, 200 mL of inoculum, 53 mL of thin stillage (replaced by distilled water in the blank group) and 147 mL of distilled water were added to achieve an inoculum volatile solid (VS) to substrate chemical oxygen demand (COD) mass ratio of 2:1. The experiments were divided into two phases: acidic shock phase and recovery phase. In the acidic shock phase, the pH of each digester was adjusted to 5.5 by 6 M HCl to mimic acidic shock for 2 days. In the recovery phase, the pH of all digesters was altered to 7.5 by 6 M NaOH to create a neutral recovery condition.

The design of the experiment included for a Control (without addition of conductive materials), Graphene (1.0 g graphene/L), Pyrochar (1.0 g pyrochar/L), and HPyrochar (10 g pyrochar/L). A group with only inoculum and distilled water in the digester was adopted as the blank group to assess the background performance (such as biomethane production, VFAs formation and COD concentration) of the inoculum (Figure S2). The choice of these concentrations was based on our previous studies, in which it was revealed that 1.0 g graphene/L showed the highest promotion effects on digestion of both glycine and ethanol possibly through the establishment of DIET.[16, 22] In this context, 1.0 g graphene/L and 1.0 g pyrochar/L were chosen to compare their effects on digestion of thin stillage. Considering the lower electrical conductivity of pyrochar, a high concentration of 10 g pyrochar/L was adopted.

Before the start of the batch experiments, all the digesters were purged by pure nitrogen to remove oxygen in the headspace and sealed immediately. All the experiments were performed at 37 ± 1 °C in a water bath. The biomethane volume during acidic shock was calculated by multiplying the biomethane content by the total biogas volume, while biomethane volume in the recovery phase was automatically counted by the AMPTS II system.

Microbial profiling

Five samples were collected for microbial community analysis, including the inoculum (before the acidic shock phase) and suspended sludge samples from the other four groups; samples of the latter were collected from one of the three parallel digesters from the sampling port on day 22. All samples were frozen at -20 °C before further analysis (detailed in Supporting Information). Significant differences in microbial community compositions between two samples based on Fisher's exact test at 0.05 level and principal component analysis were analysed on the Majorbio Cloud Platform (www.majorbio.com). The raw sequence data were deposited into NCBI Sequence Read Archive under accession code SRP258370.

Analytical methods

TS and VS were determined according to methods outlined in a previous study.[16] A gas chromatograph (GC, Agilent 7890B, USA) equipped with a flame ionization detector and a DB-FFAP column was used to quantify the compositions of biogas and VFAs (details in Supporting Information).[24] Before GC analysis, the liquid samples were acidified with 12% orthophosphoric acid (v/v). Soluble carbohydrate content was determined using the anthrone-sulfuric acid method with glucose as standard.[25] The elemental composition of thin stillage was analysed using an elemental analyser (Exeter Analytical, CE 440, USA). COD, total nitrogen (TN) and ammonia nitrogen (NH_3-N) were measured using appropriate test kits (Hach, USA). The pH was measured by a pH meter (F20, METTLER TOLEDO, Switzerland).

Experimental data evaluation

Thermodynamic calculation

The change in the Gibbs free energy ($\Delta G'$) of propionate oxidation was calculated according to Eq. (1).

$$\Delta G' = \Delta G^{0'} + RTLn\frac{[Acetate] \cdot pCO_2 \cdot pH_2{}^x}{[Propionate]} \quad (1)$$

Where $\Delta G^{0'}$ (kJ/mol) is the free energy change under the following conditions (T = 298.15 K, pH = 7, Pressure = 1 atm, and [Reactants] = 1 M); R is the universal gas constant (8.315 J/mol/K); T (K) is the absolute reaction temperature; [*Acetate*] and [*Propionate*] represent the respective concentration of acetate and propionate in the reaction (mol); pCO_2 is the concentration of carbon dioxide in the reaction (atm); pH_2 is the concentration of hydrogen in the reaction (atm); x represents number of molecules in the reaction. Thermodynamic calculation was conducted using values obtained from the graphene group on day 6, in which [*Acetate*], [*Propionate*] and pCO_2 was 5.1 mM, 9.4 mM and 0.44 atm, respectively.

CH_4 production modelling and statistical analysis

CH_4 production data in the recovery phase were fitted by the modified Gompertz model,[16] as shown in equation Eq. (2). The parameters (P, maximum CH_4 yield potential, mL/g COD; R_{max}, maximum CH_4 production rate, mL/g COD/day; λ, lag time of CH_4 production, day; t, time, day) were analysed using the Origin software (8.5, Origin, USA). Significance analysis of experimental data was carried out as previously described.[26]

$$M = P \cdot exp\left\{-exp\left[\frac{R_{max} \cdot e}{P}(\lambda - t) + 1\right]\right\} \quad (2)$$

Results and Discussion

Theoretical analysis of interspecies transfer via direct electron or indirect hydrogen

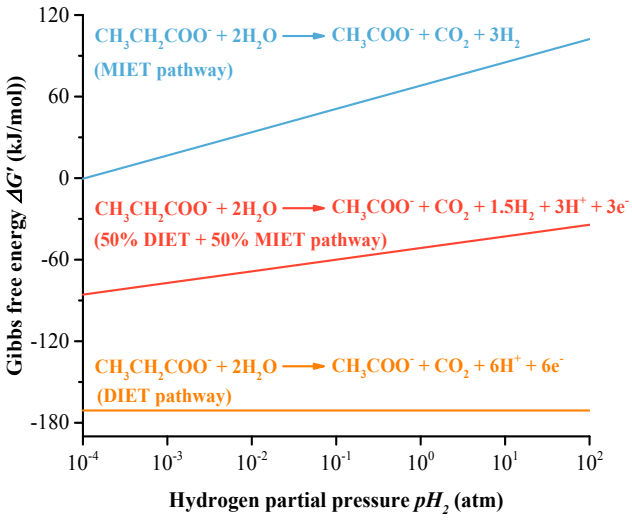


Figure 1. Thermodynamic comparison of propionate oxidation via mediated interspecies electron transfer (MIET) and direct interspecies electron transfer (DIET) (pH = 7, T = 298.15 K, [*Propionate*] = 9.4 mM, [*Acetate*] = 5.1 mM, pCO_2 = 0.44 atm).

Table 1 Reactions and changes in Gibbs free energy values of propionate conversion to methane in different pathways.

Process	Reaction	$\Delta G^{0\prime *}$ (kJ/mol)
Electron-generating reaction	MIET: $CH_3CH_2COO^- + 2H_2O \rightarrow CH_3COO^- + CO_2 + 3H_2$	+71.61
	DIET: $CH_3CH_2COO^- + 2H_2O \rightarrow CH_3COO^- + CO_2 + 6H^+ + 6e^-$	–167.37
Electron-accepting reaction	MIET: $3H_2 + 0.75CO_2 \rightarrow 0.75CH_4 + 1.5H_2O$	–98.02
	DIET: $6H^+ + 6e^- + 0.75CO_2 \rightarrow 0.75CH_4 + 1.5H_2O$	+140.96
Acetate conversion reaction	$CH_3COO^- + H^+ \rightarrow CH_4 + CO_2$	–35.91
Overall	$CH_3CH_2COO^- + H^+ + 0.5H_2O \rightarrow 1.75CH_4 + 1.25CO_2$	–62.32

* Values are computed under the conditions of T = 298.15 K, pH = 7, Pressure = 1 atm, and [Reactants] = 1 M based on tabulated data by Madigan et al..[27]

Propionate, a typical intermediate for carbon and electron flow in digestion of organic material was chosen to investigate the syntrophic interactions in energy-limited methanogenic biosystems,[28, 29] and to compare mediated interspecies electron transfer (MIET) and direct interspecies electron transfer (DIET). Complete degradation of propionate to CH_4 and CO_2 needs the well-established connections between syntrophic acetogens and methanogens; these relationships determine the efficiency of the electron transfer. Figure. 1 shows that overall propionate oxidation to acetate is thermodynamically more favourable via DIET than via hydrogen transfer.

In the MIET pathway, acetogenic bacteria convert propionate into acetate and H_2 (Table 1). Under the conditions specified (namely pH = 7, T = 298.15 K, [*Propionate*] = 9.4 mM, [*Acetate*] = 5.1 mM, pCO_2 = 0.44 atm; values are collected from experimental data), the reaction is thermodynamically favourable only when the concentration of H_2 is below 1.0×10^{-4} atm, at which the Gibbs free energy change equals to 0. This makes propionate oxidation to acetate more vulnerable as an increase in hydrogen partial pressure would result in the increase in the Gibbs free energy change to a level that makes the reaction unfavourable.

In the DIET pathway, the change in hydrogen partial pressure does not affect the Gibbs free energy as it does not involve exchange of diffusible molecules among syntrophic partners. If both MIET and DIET pathways take place, the Gibbs free energy change depends on the proportion of electrons transferred through MIET or DIET. As an example, if only half of electrons produced from propionate oxidation to acetate are transferred through DIET at a hydrogen partial pressure of 1.0×10^{-4} atm, approximate 85 kJ/mol of energy advantage can be expected compared with that of complete MIET (Figure 1). It should be noted that the overall change in Gibbs free energy values of propionate conversion to methane between DIET and MIET pathways is theoretically the same.

The computed maximum electron carrier flux during propionate oxidation to methane demonstrated the advantage of DIET as compared to MIET (hydrogen diffusion), indicating that the theoretical difference between them was significant with a 10^6 factor.[29] It is worth mentioning that the calculations were based on numerous assumptions, several of which cannot be said to be 100%

precise. For example, neither the heat lost nor the energy demand for growth and maintenance of microorganisms was considered during computing. However, these numbers are small and as such the significant difference between two fluxes may still be said to be a distinct kinetic merit of DIET. Given the advantages of electron carrier flux and thermodynamics, DIET is preferable to MIET in terms of facilitating propionate oxidation among syntrophic partners in AD.

Performance of biomethane production from thin stillage with conductive materials amendment

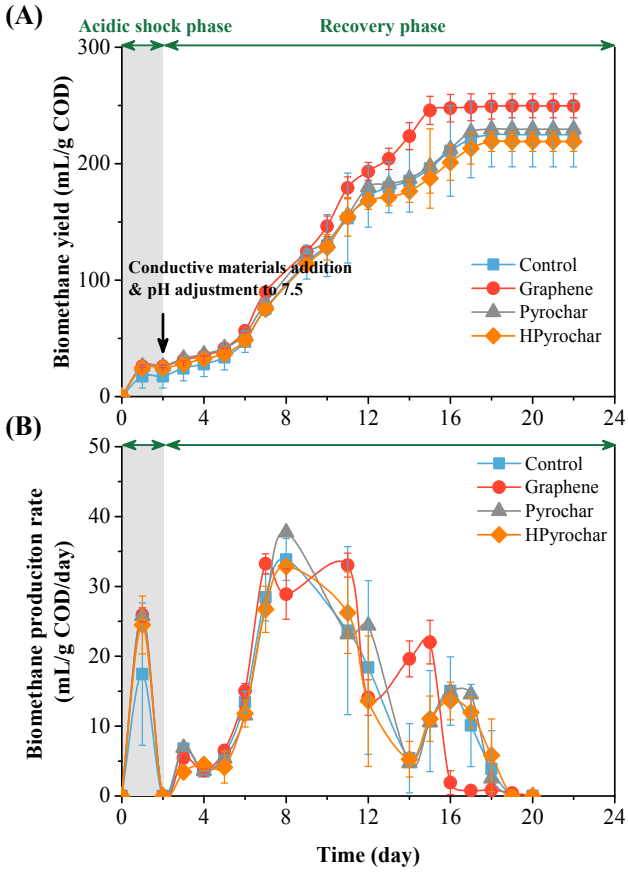


Figure 2. Effects of conductive materials amendment on the performance of (A) biomethane yield and (B) production rate during anaerobic digestion of thin stillage after acidic shock.

The effects of conductive materials addition on the performance of biomethane yield and production rate from thin stillage are illustrated in Figure 2. During the acidic shock phase, biomethane production increased slightly on day 1, and remained unchanged on day 2, indicating that the methanogenesis process was completely inhibited by acidic shock. In the recovery phase, the

biomethane yield of the control group reached 225 mL/g COD after 18 days of digestion. With the introduction of graphene (1.0 g/L), biomethane yield increased to 250 mL/g COD, an increase of 11.0% compared with the control. Lin et al. revealed that 1.0 g graphene/L had the most positive effect generating 13.8% higher levels of CH_4 production as compared to the control in digestion of ethanol.[22] Similar results were found in another work,[16] where protein-derived amino acid was used as the substrate. However, here the addition of pyrochar did not lead to any significant effects on biomethane yield ($p > 0.05$), both at low and high dosages (1 g/L and 10 g/L), generating between 219 and 230 mL/g COD. Luo et al. applied pyrochar with different particle sizes to evaluate the effect on AD facing various acidic stress levels.[30] Results indicated that compared to the control, pyrochar adoption shortened the lag-phase for methanation in all cases, but it also brought negative impacts as the total biomethane yield was reduced by between 2.5% and 17.5%.[30] Similarly, Wang et al. demonstrated that 2 g/L to 15 g/L of pyrochar could reduce the lag time when treating a mixture of dewatered activated sludge and food waste, but there was no increase in biomethane yield.[31] These findings are consistent with the results in this study, which showed that 1.0 g/L and 10 g/L of pyrochar shortened the lag time by 18.1% and 12.2% (Table 2), respectively, while biomethane yield was not obviously increased.

Without the introduction of conductive materials, the biomethane production rate peaked on day 8 at 33.85 mL/g COD/day, as shown in Figure 2B. Among all cases, the highest peak production rate was obtained with a pyrochar introduction of 1.0 g/L, an 11.5% increase compared with that of the control, reaching 37.73 mL/g COD/day on day 8. Despite the greatest promotion effects on the cumulative biomethane yield, graphene only led to a highest peak production rate of 33.23 mL/g COD/day on day 7, no significant difference compared with that of the control ($p > 0.05$). However, during the latter recovery phase, the biomethane production rate with graphene addition still maintained a relatively higher level compared with other groups. For instance, the corresponding value of the graphene group was 22.03 mL/g COD/day on day 15, which was significantly higher than that of other groups ($p < 0.05$) which had a level of approximately 10 mL/g COD/day.

Table 2 presents the simulated parameters of biomethane production using the modified Gompertz model. The potential biomethane yield of the graphene group increased by 11.5% whilst the peak biomethane production rate enhanced by 17.9% compared with the control. Comparatively, pyrochar addition shortened the lag time as described earlier but did not improve the total biomethane production. These results revealed that the amendment of graphene could resist acidic shock and thus stabilize and enhance the AD performance of thin stillage, but pyrochar had no evident effect in terms of recovering biomethane yield.

Table 2 Estimated parameters describing biomethane production using the modified Gompertz equation.

Group	$P_{measured}$ (mL/g COD)	P (mL/g COD)	R_m (mL/g COD/day)	λ (day)	T_m (day)	Adjusted R^2
Control	224.92	239.82	20.49	3.37	7.69	0.9916
Graphene	249.73	267.32	24.15	3.36	7.44	0.9878
Pyrochar	229.54	247.7	19.4	2.76	7.46	0.9911
HPyrochar	218.92	234.58	18.9	2.96	7.54	0.9908

Note: Control: no conductive materials added; Graphene: 1 g graphene/L; Pyrochar: 1 g pyrochar/L; HPyrochar: 10 g pyrochar/L; $P_{measured}$: experimental methane yield; P: maximum methane potential; R_m: maximum methane production rate; λ: lag-phase time; T_m: peak production time.

The proposed attribution of positive effects on biomethane production in the graphene group was that DIET was stimulated in the presence of highly conductive graphene, possibly acting as electron conduits between syntrophs and methanogens. In general, the electrical conductivity of pyrochar is in a range of several to ten siemens per centimetre (S/cm),[32, 33] while the conductivity of graphene (generally tens to hundreds S/cm) is several orders of magnitude higher than that of pyrochar.[34, 35] Compared with pyrochar, the significant higher conductivity of graphene might be more beneficial to establishing a strong syntrophic relationship and to triggering efficient DIET between

electron donating and accepting microbes, thus enhancing cumulative biomethane production. DIET was reported to proceed via three possible pathways, namely; redox mediators, e-pili adhered with cytochromes, and conductive materials.[14, 36] Redox mediators and e-pili are also called electron shuttles. The properties of surface structure, surface chemistry and redox mediators of pyrochar have been highlighted in other studies.[37] Yu et al. and Wang et al. proved that pyrochar played a critical role in alleviating external inhibition caused by refractory compounds due to its surface redox-active moieties that might favour DIET.[32, 38] However, in this study, the role of pyrochar serving as an electron shuttle mechanism might not be sufficient to trigger efficient DIET because the unique role of electron conduits cannot be substituted by electron shuttles.[39] These findings lead to a plausible conclusion that the promoted biomethane production resulted from the electron conduit function transferring electrons between microbes rather than the electron shuttle. Meanwhile, surface functional groups of pyrochar might play a positive effect on the recovery of AD systems with acidic shock in the initial period, which shortened the lag time, but due to its low conductivity, pyrochar failed to generate an efficient DIET process subsequently.

Variations of COD, VFAs and pH levels during digestion of thin stillage

The VFAs formation and COD concentration over time are shown in Figure 3. VFAs accumulated rapidly in the initial period and peaked on day 4 in all groups.

For the control group, the total maximum VFAs concentration was 1724 mg/L, in which acetate, propionate and butyrate were dominant, making up 28.5%, 36.7% and 26.8% of the total VFAs, respectively. From day 9 to 14, the total amount of VFAs reduced from 842 to 650 mg/L and more than 90% of VFAs were in the form of propionic acid. By day 22 the VFAs had degraded to 20.3 mg/L.

The graphene group showed similar trends in the initial days (day 0 to 9), during which the amount of VFAs peaked at 1805 mg/L on day 4 and reduced to 815 mg/L by day 9. However, the subsequent degradation of VFAs in the graphene group significantly accelerated, decreasing to 373

mg/L on day 14, 42.6% lower compared than that of the control ($p < 0.05$). Meanwhile, propionic acid was the dominant VFA, accounting for 97.9% of total VFAs; the residual was isobutyric acid.

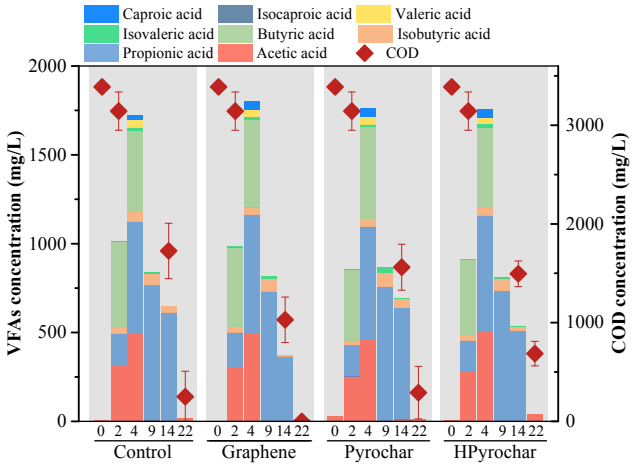


Figure 3. Effects of conductive materials amendment on the concentrations of volatile fatty acids (VFAs) and chemical oxygen demand (COD) during anaerobic digestion of thin stillage with acidic shock (0, 2, 4, 9, 14, and 22 represent the corresponding day).

Pyrochar amendment did not significantly alter the proportion of VFAs as compared with the control group regardless of the concentration; 1 g/L or 10 g/L. Similar to the control group, the total amount of VFAs in Pyrochar and HPyrochar groups on day 4 achieved the maximum value of 1763 and 1758 mg/L, respectively. Thereafter, these figures decreased to 696 and 532 mg/L on day 14, respectively. The results indicated that pyrochar amendment did not show a significant effect on the formation of VFAs during the digestion of thin stillage with acidic shock.

The COD concentration in different groups over time is presented in Figure 3. During the acidic shock phase, the amount of COD slightly reduced from 3388 mg/L on day 0 to 3143 mg/L on day 2; this is attributed to the consumption of easily degraded compounds. For the graphene group, the concentration of COD was 1029 mg/L on day 14, which is 40.4% lower than that of the control, and this figure reduced to 0 by day 22. On day 22, the addition of pyrochar presented no positive impact on the degradation of COD from thin stillage, since the amount of COD remained at 250 mg/L in the control group while this figure was 291 mg/L and 687 mg/L in Pyrochar and HPyrochar groups, respectively. The higher final COD concentration in HPyrochar group possibly resulted from the

strong absorption capacity of pyrochar, especially under high dosages.[40] The COD concentration over time suggested a good agreement with the observed biomethane yield and VFA concentration during the digestion of thin stillage.

To get further insights into VFAs degradation, the variation of butyric acid and propionic acid during AD is shown in Figure 4A.

Butyric acid in the control group accumulated rapidly in the first day, reaching 478 mg/L. Thereafter, it varied slightly during day 1 to 6 but was totally degraded on day 9. The addition of graphene had no apparent effect on the formation and degradation of butyric acid, which showed a similar trend to the control group.

Propionic acid climbed gradually and reached a peak level of 762 mg/L on day 9 in the control group and of 747 mg/L on day 11 with the addition of graphene. The largest differentiation in terms of the amount of propionic acid between the control and graphene groups was obtained on day 14, during which propionic acid in the graphene group was 365 mg/L while this figure was 613 mg/L in the control group. Thereafter, propionic acid was totally degraded by day 18 in both groups. The results indicated that graphene amendment accelerated the degradation of propionic acid, which is consistent with the expected outcome that propionate oxidation via DIET pathways is thermodynamically more favourable than via hydrogen diffusion. However, the time requirement for complete degradation of propionic acid was much higher than for butyric acid, which aligns with similar observations by other studies.[19, 41] One possible explanation for this based on thermodynamics, is that the energy barrier of converting propionate to acetate ($\Delta G^{0\prime}$ = +71.61 kJ/mol, Table 1) is much higher than that of converting butyrate to acetate ($\Delta G^{0\prime}$ = +48.3 kJ/mol).[31] Therefore, the syntrophic oxidation of propionate through the MIET pathway needs much lower hydrogen partial pressure than for the butyrate oxidation.[19] Due to thermodynamic fundamentals, butyrate degrades much faster than propionate. Butyrate was consumed completely by day 9 in both groups without apparent accumulation. Conversely, due to the higher energy barrier, apparent accumulation of propionate occurred in both groups. However, the addition of graphene, which is capable of altering the metabolic pathway to a DIET process and alleviating potential hydrogen partial pressure,[41] enhanced

the degradation of propionate. The results suggested that graphene addition had a significant effect on propionate degradation but no obvious effect on butyrate during digestion of thin stillage.

After the acidic shock, the pH of all groups was adjusted to 7.51 ± 0.03 and different conductive materials were introduced into corresponding groups. As can be seen from Figure 4B, the pH of all groups varied slightly with similar trends during the recovery phase. The pH value of the Control, Graphene, Pyrochar, and HPyrochar groups after AD was recorded as 7.25, 7.24, 7.27, and 7.20, respectively ($p > 0.05$). This indicated that pH did not govern the performance of thin stillage digestion with conductive materials amendment.

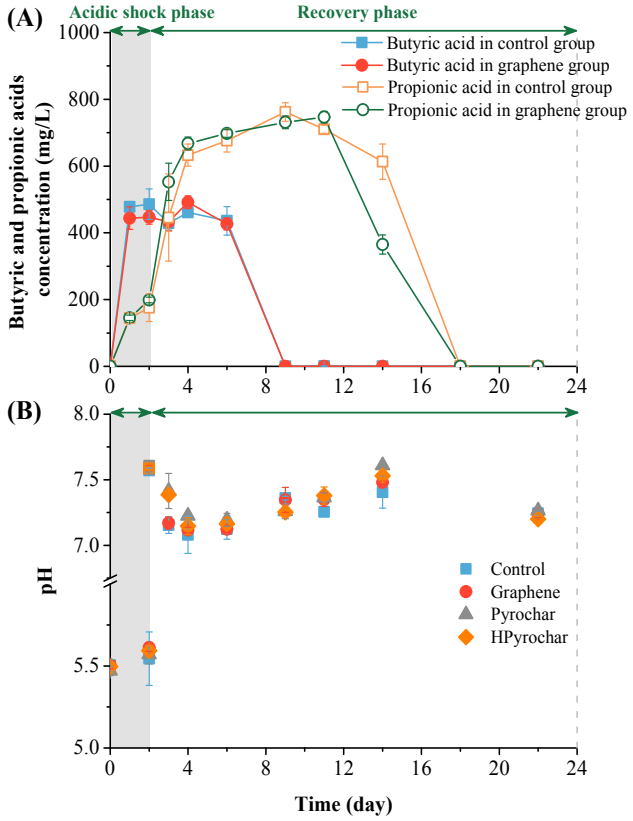


Figure 4. Variations of (A) butyric and propionic acids and (B) pH levels during anaerobic digestion of thin stillage with acidic shock.

Response of microbial community structures to conductive materials amendment

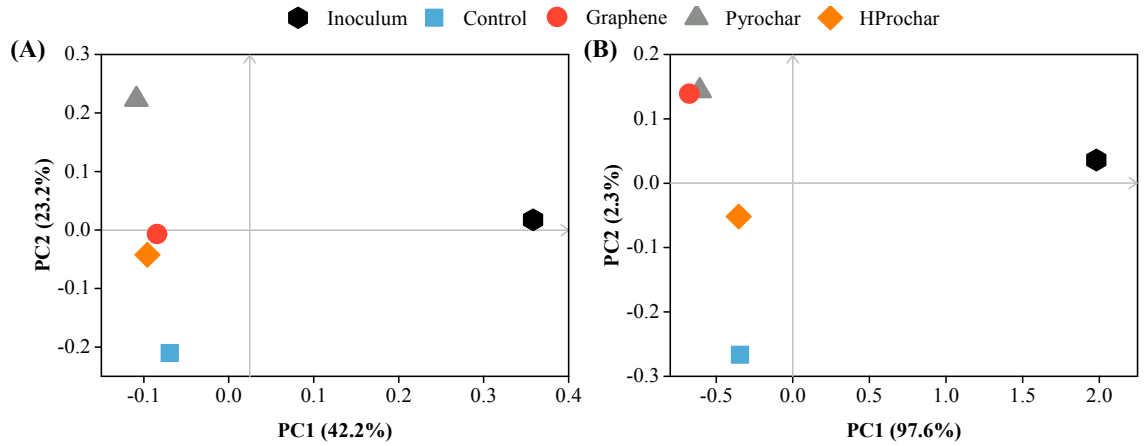


Figure 5. Principal component analysis of (A) bacterial and (B) archaeal community based on the OTU (PC: principal component).

Microbial community analysis was conducted to provide some insights into the effect of conductive materials on digestion of thin stillage with acidic shock, by employing high-throughput sequencing. Principal component analysis (PCA) of the bacterial community was conducted to evaluate the microbial diversity based on operational taxonomic units (OTUs) (Figure 5). For bacteria, the first axis and second axis accounted for 42.2% and 23.2% of data variance, respectively (Figure 5A). PCA of the bacterial and archaeal community showed varying levels of separation following amendment with conductive materials (Figures 5A and B). Amendment with pyrochar (10 g/L) and graphene (1 g/L) resulted in bacterial populations displaying similar separation patterns, with pyrochar amendment (1 g/L) resulting in the strongest separation from the nonamended control group. PCA of the archaeal community revealed that pyrochar (1 g/L) and graphene (1 g/L) amendment led to the strongest similar separation patterns relative to the control group (Figure 5B). These results clearly indicated that pyrochar addition (1 g/L) resulted in the most marked changes in both bacterial and archaeal community structures relative to the nonamended control group, while graphene amendment appeared to only induce apparent modifications in the archaeal community. This may be as a result of the previously highlighted properties of pyrochar, such as surface functional groups and

rough surface structure, which may promote colonization and biofilm formation of microbes when compared with graphene.[37, 42]

Compared with the inoculum, the most significant increase in abundance in the control group was obtained at genus *Fermentimonas*, which rose from 0.2% to 10.8%, indicating its unique role during digestion of thin stillage (Figure 6A). *Fermentimonas* has been reported as the dominant genus in digesting numerous complex substrates and shown to be associated with the hydrolysis and acidogenesis of AD process.[43] Moreover, it can convert substrates containing polysaccharides and proteins to easy-degraded VFAs, which are the main substrates for methanogens.[44] This is supported by "*in silico*" analysis of the genome of the *Fermentimonas caenicola* strain which indicates that it contains a large number of genes encoding carbohydrate-active enzymes potentially involved in hydrolysis and it has also been shown to be involved in propionic acid fermentation and to contain genes potentially encoding the acrylyl-CoA pathway also involved propionic acid fermentation.[45]

Other genera such as *Aminobacterium* (3.89%), *Tepidanaerobacter* (4.8%) and an OTU in the family *Lentimicrobiaceae* (5.4%) also became predominant over time in the control group when compared with the inoculum. Members of the *Aminobacterium* genus are typically amino-acid-degrading bacteria that can ferment various amino acids when co-cultured with hydrogenotrophic methanogens.[16, 46] They are also known to be particularly resilient, and increased *Aminobacterium* levels have previously been reported following heat-shock treatments within the acidification stage of a two-stage reactor system for AD and biomethanation of grass.[47] *Tepidanaerobacter* has been revealed as the syntrophic acetate-oxidizing bacteria (SAOB) that generally cooperates with hydrogen-utilizing methanogens such as *Methanosarcina*.[43] Microbial groups belonging to family *Lentimicrobiaceae* are capable of the hydrolysis of complex polysaccharides such as food waste-recycling wastewater[48] and high-strength starch-based wastewater.[49] These findings suggest that new syntrophic relationships amongst the above-mentioned bacterial genera and potential methanogens are likely to have been established to efficiently convert thin stillage to methane.

Significant variations were also observed in the archaeal community during the digestion of thin stillage (Figure 6B). Compared with the inoculum, the most apparent modification observed from the

control group was in members of the genus *Methanosarcina*, which increased from 4.3% to 64.6% of the total relative abundance. *Methanosarcina* has been widely reported under mesophilic conditions in AD treating various feedstock.[14, 50] Aside from the documented excellent tolerance to external pressures such as high pH or ammonia,[51] members of this genus have metabolic pathway that can be both acetoclastic and hydrogenotrophic. Previous studies also suggested that *Methanosarcina* can directly receive electrons for CO_2 reduction to methane.[52] These findings highlight the vital role of *Methanosarcina* in digesting thin stillage under condition of acidic shock.

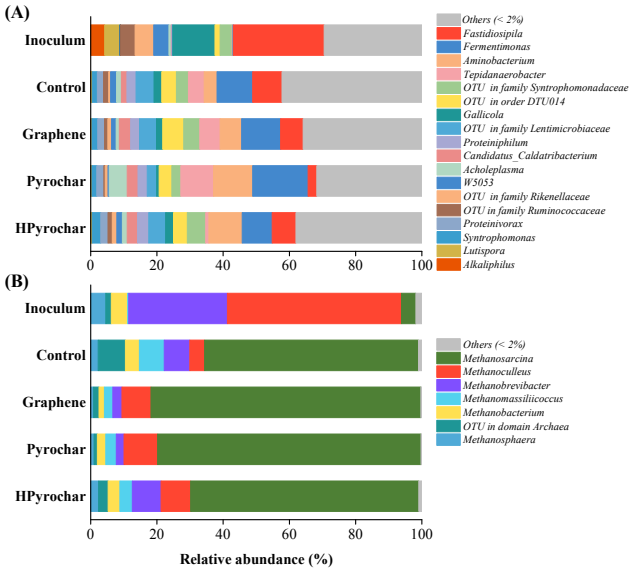


Figure 6. Taxonomic composition of Bacteria (A) and Archaea (B) at genus level. Genera with lower abundances than 2% are classified into "*Others*".

With the addition of conductive materials, the overall bacterial and archaeal community structure varied depending on the operational strategy. Considering the enhancement in biomethane production, a detailed analysis of the differential between the control and graphene groups was performed to help provide insights into potential mechanisms (Figure 7). Following the amendment of graphene, the most significant modification in bacterial community structure was observed in the genus *Aminobacterium*, which increased from 3.9% to 6.5% in relative abundance (Figure 7A), indicating their potential role in accelerating the process of hydrolysis and acidogenesis during thin stillage

digestion.[46] There was also a significant increase in the relative abundance of an OTU in the order *DTU014* in the graphene group, which increased from 4.4% to 6.4% (Figure 7A). Members of this order have been identified as potential syntrophic bacteria that can establish magnetite-mediated DIET with methanogens such as *Methanosarcina*. They have been reported as the dominant bacteria in both propionate and butyrate treatments and suggested to be able to perform conductive materials-mediated DIET under stressful environments (such as with high levels of ammonia).[53]

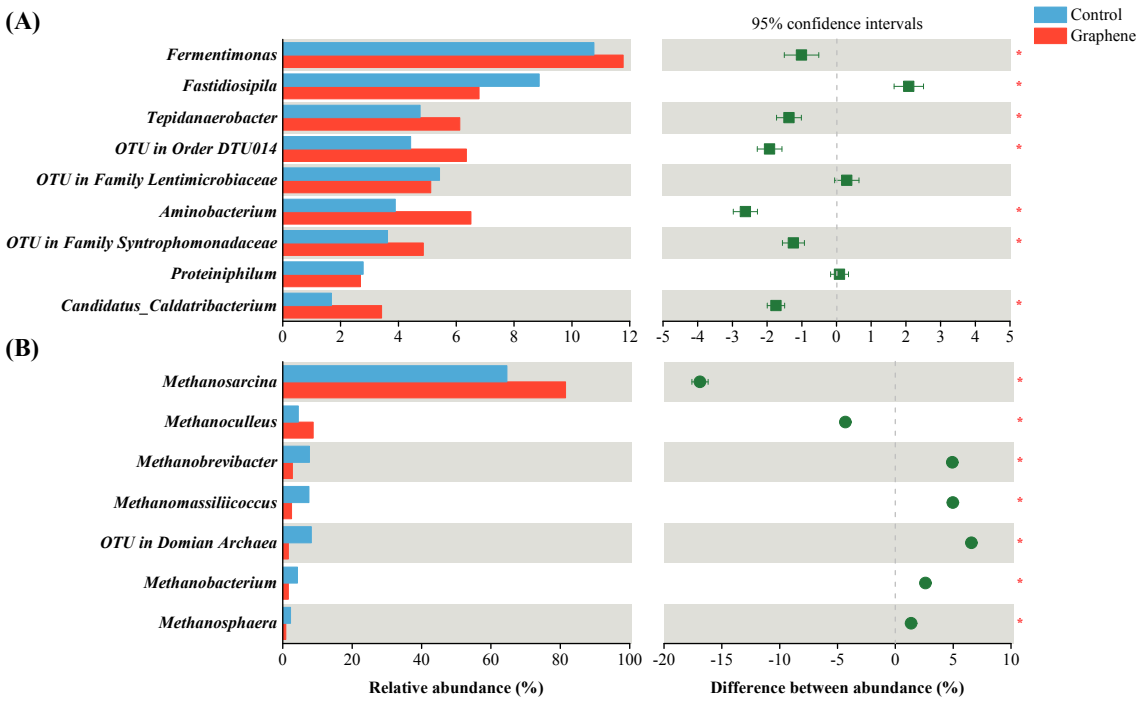


Figure 7. Significant difference between the control and graphene group on relative abundance from 16S rRNA gene sequencing result (* represents p-values less than 0.05).

The relative abundance of *Methanosarcina* also significantly increased from 64.6% in the control group to 81.5% in the graphene group (Figure 7B). *Methanosarcina* have been clearly demonstrated to be responsible for performing DIET following supplementation with conductive materials.[36, 54] However, the capacity of most syntrophs and methanogens in functioning DIET still remains unclear. Microbes, which are enriched by conductive materials and gain prominence in AD systems are generally considered as the potential syntrophic bacteria involved in DIET.[36] However, the complexity of real organic wastes makes it difficult to find possible DIET partners in AD when compared to simple substrates. Among the genera where significantly increases were observed

(Figure 7), only the OTU in Order *DTU014* has previously been reported to function in DIET.[53] In this context, given that the OTU in Order *DTU014* was dramatically enriched with graphene addition when compared to the control, it seems likely that this genus is responsible for DIET functioning and stimulation of the degradation of VFAs by cooperating with methanogens, namely *Methanosarcina*, particularly in the degradation of propionic acid. However, the participation of MIET cannot be completely ruled out since *Methanosarcina* are mixotrophic and are also able to accept electrons from H_2. The increased relative abundance in the genus *Tepidanaerobacter*, which as previously mentioned has the potential to serve as SAOB with *Methanosarcina*,[55] indicated the likelihood of enhanced syntrophic connections via traditional MIET.

Clearly, graphene amendment attributed to the enhancement of biomethane production. Compared with the control, graphene addition resulted in changes in the microbial community which are likely to have promoted connections between syntrophs and methanogens via both MIET and DIET. Moreover, the high electrical conductivity property of graphene itself might also be a critical factor in accelerating electron transfer potentially through acting as an electron conduit rather than electron shuttle between order *DTU014* (VFA oxidizing bacteria) and the genus *Methanosarcina* (methanogens).

Table 3 Comparison of the effect of conductive materials amendment on anaerobic digestion facing external stress.

Conductive materials	Feedstock	Temperature (°C)	Operation mode	External stress	Methane production increase [a]	Highlighted microbes	Reference
Carbon cloth	Butanol	37	Semi-continuous	Acidic stress: pH decreased from 7.0 to 5.0 gradually	+59.0%	*Geobacter*, *Methanosaeata*	Zhao et al.[13]
Pyrochar	Glucose	37	Batch	Acidic stress: 2–8 g glucose/L	-2.5% – -17.5%	*Syntrophomonas, Methanobacterium, Methanosarcina*	Luo et al.[30]
Magnetite	Acetate	37	Batch	Ammonia stress: 5 g NH_4^+–N/L	+3.0%	*Geobacteraceae*, *Methanosarcinaceae*, *Methanobacteriaceae*	Zhuang et al.[56]
Pyrochar	Glucose	35	Batch	Ammonia stress: 7 g NH_4^+–N/L & acidic stress: 6 g glucose/L	+1.7% – +12.1%	*Methanosaeta, Methanosarcina*	Lü et al.[17]
GAC	Blackwater	35	Batch	Ammonia stress: 2.8 g NH_4^+–N/L	+9.0% – +53.4%	*Geobacteraceae*, *Clostridiales*, *Methanosarcina*	Florentino et al.[18]
Pyrochar	FW + SS	55	Semi-continuous	High OLRs: 1.6–5.4 g VS/L/d	+16.0% − +55.2% [b]	*Tepidimicrobium*, *Methanothermobacter*	Wang et al.[57]
Pyrochar	Poultry litter	37	Batch	High OLRs: total solid contents from 5% – 20% [c]	-4.7% – -14.0%	*Methanosaetaceae*	Indren et al.[15]
Pyrochar	FW + WAS	55	Batch	High OLRs: feedstock/seed sludge (VS/VS) mass ratio of 1.5–3	-3.8% – -7.9%	*Methanosaeta*, *Methanosarcina*	Li et al.[58]
Graphene	Thin stillage	37	Batch	Acidic shock: pH = 5.5 for 2 days	+11.0%	OTU in order *DTU014, Methanosarcina*	This study
Pyrochar	Thin stillage	37	Batch	Acidic shock: pH = 5.5 for 2 days	-2.7% – +2.1%	*Methanosarcina*	This study

[a] Compared with the control group; [b] the control digester was completely inhibited when the OLR was over 2.7 g VS/L/d; [c] adjusted by water

FW: food waste, SS: sewage sludge WAS: waste activated sludge, GAC: granular activated carbon, Blackwater: urine and feces, NA: not analysed, OLR: organic loading rate

Comparison of conductive materials amendment in anaerobic digestion

This study demonstrated that graphene addition could help resist acidic shock and stabilize biomethane production from thin stillage, whereas the effect of pyrochar amendment was not significant in increasing cumulative biomethane yield though it did aid in reducing the lag time. The addition of conductive materials has been suggested as a feasible strategy to enhance biomethane production through stimulating DIET, and has also been adopted to enhance the robustness of AD facing external stress, thereby leading to a more stable biomethane production performance.

Table 3 summarizes some conductive materials applied to enhance the DIET pathway under stressed AD conditions. Methane production was enhanced by 1.7% – 59% depending on the types of conductive materials and external stress levels (Table 3). However, some conductive materials also had a negative effect on AD performance with biomethane yield being reduced by 2.5% – 31.3% (Table 3). Zhao et al. showed the introduction of carbon cloth could stabilize the AD system under acidic stress by shifting the predominant working mode from traditional MIET to more efficient DIET.[13] The primary microbial participants in DIET, namely *Geobacter* and *Methanosaeata*, were enriched in such environment.[13] Conversely, Luo et al. observed a negative impact of pyrochar amendment on biomethane yield encountering acidic stress from high load of glucose.[30] Other traditional conductive materials (such as pyrochar, magnetite and GAC) have also been assessed with respect to their potential role in resisting ammonia inhibition during AD. Zhuang et al. and Florentino et al. reported that the performance of methanogenic digesters exposed to different levels of ammonia was improved with the addition of magnetite and GAC.[18, 56] The potential syntrophs and methanogens (such as *Geobacteraceae* and *Methanosarcinaceae*) that could function in a DIET system were both highlighted in their studies.[18, 56] Operational inhibition of the AD systems, such as high OLRs, was also investigated with the supplement of carbon-based materials.[15, 58] Li et al. pointed out that pyrochar addition facilitated DIET in syntrophic oxidation of butyrate and acetate under high OLRs of food waste and waste activated sludge, whereas simultaneously it was observed to hamper biomethane production.[58] This might be attributed to the reduced bioavailability during digestion due to the non-selective absorption capacity of pyrochar.[40]

In this study, pyrochar addition reduced lag phase time of AD, but did not apparently improve biomethane production from thin stillage under both low and high dosages. Pyrochar, especially produced through the valorisation of organic wastes such as digestate and wood waste, has the potential to be applied as an additive in AD to boost microbial interactions and enhance AD performance. However, the effects of pyrochar on AD highly depend on its properties.[58] Some studies have reported that enhanced AD performance resulted from the highlighted properties of pyrochar, such as surface functional groups, conductivity, buffer capacity, and surface area.[32, 59] In this study, pyrochar was produced from wood waste at a pyrolysis temperature of 700 °C, which has been suggested as the threshold temperature for woody biomass-derived pyrochar to provide electron transfer function from electron shuttles to electron conduits, resulting in a faster direct electron transfer.[60] Cruz Viggi et al. added three different kinds of pyrochar to enhance the AD peroformance of food waste; significant promotional effects on biomethane production rate were observed in all pyrochar ammended groups.[61] Interestingly, results demonstrated a strong correlation between the electron donating capacity related to their surface functional groups and the enhancement effect on methanogenic activity.[61] Graphitic carbon generally has high electrical conductivity.[34, 35] The importance of electrical conductivity in stimulating DIET has been widely recognized by other studies.[21, 62] Different types of conductive materials with different electrical conductivity can lead to distinguishing influence on AD performance.[63] However, enhancement of AD was not always observed.[62] Possible explanations could be that a certain threshold of electrical conductivity needs to be overcome to induce efficient DIET in methanogenic digesters.[21] The significant difference in electrical conductivity between pyrochar and graphene may be the critical factor influencing biomethane yield from thin stillage with acidic shock. Surface complexation of pyrochar might play a positive role on digestion of thin stillage in the initial period, but did not prove to have an obvious impact on the digestion of thin stillage subsequently. It should be noted that it is difficult to draw direct conclusions in terms of determining roles of conductive materials when different types of materials are used in AD systems treating complex organic substrates. The same conductive materials can show variable effects on digestion of different sustrates. In this study, 1.0 g/L of graphene

amendment increased biomethane production by 11% from thin stillage. However, different impacts of graphene on AD were observed in other studies; 1.0 g/L of graphene only increased biomethane yield from protein-derived glycine by 4.2% but promoted peak biomethane production rate by 28.0%.[16] Similarly, the effect of pyrochar can differ depending on the substrate. Chen et al. added wood-derived pyrochar to digesters to assess its potential role in enhancing digestion of seaweed; the optimal promotional effect on biomethane production from *L. digitata* (17%) was obtained with the addition of 0.25 g biochar/g VS seaweed while a reduction of biomethane yield from *S. latissima* (6%) was found using the same dosage of pyrochar.[23] These findings tend to conclude that not only the added conductive materials but the properties of the substrates digested impact on the precise change in AD performance with addition of conducive materials. Further work is needed to investigate the correlations between the electrical conductivity of conductive materials and performance during AD of thin stillage with acidic shock.

The target for advanced biofuels in the recast Renewable Energy Directive for 2030 in the transport sector is only 3.5%, which highlights the lack of advanced biofuel technologies. AD technology integrated with DIET offers a potential way to meet this target considering its stabilization and promotion effects on biomethane production (ca. 11% increase achieved in this study). Specifically, the addition of conductive materials may represent a reliable strategy to alleviate the impact of external stress in AD systems, typically due to the establishment of DIET, which can enhance the robustness of AD systems and ultimately promote biomethane production from various wet organic feedstocks. The use of conductive materials such as graphene (typical cost €644/kg in the form of nanosheets[23]) may affect their practical application especially at industrial scale. However, reuse through strategies such as the design of reactor configuration to retain conductive materials in bioreactors may make the process economically feasible. Pyrochar (average price ca. €3/kg) has potential as a cheap alternative to expensive conductive materials.[23] Pyrochar produced at different temperatures from different raw feedstocks has a range of properties.[64] The addition of a suitable kind of pyrochar in anaerobic digesters can shorten the lag time of digestion and promote biomethane yield, thereby reducing the cost of the developed AD systems. Through the integration of pyrolysis

and AD technology in a circular cascading bio-based system, a slight increase in net gain energy and a significant reduction in digestate mass flow can be achieved.[23] Considering AD is one of the most effective renewable energy producing biotechnologies, with thousands of biogas plants worldwide, for example, Germany has more than 10,000 biogas plants,[65] the results obtained in this study would benefit the achievement of advanced biofuels production target.

Conclusion

This study demonstrated that the addition of graphene could help stabilize AD of thin stillage after acidic shock, presumably due to direct interspecies electron transfer (DIET). Graphene amendment (1.0 g/L) promoted biomethane yield by 11.0% compared with the control and accelerated the degradation of propionic acid. Thermodynamic calculations indicated that if only half of electrons produced from propionate oxidation are transferred through DIET, approximate 84 kJ/mol of an energy advantage can be expected compared with that of indirect hydrogen transfer. In comparison, pyrochar addition (1.0 g/L and 10 g/L) shortened lag time but failed to enhance biomethane yield. Microbial analysis revealed that DIET responsible syntrophs (OTU in the order *DTU014)* and archaea *Methanosarcina* were significantly enriched with graphene addition, suggesting a potentially important role in stabilizing and improving biomethane production through functioning efficient DIET.

Supporting Information

Method for microbial profiling; Method for biogas composition analysis; Method for soluble COD concentrations of pyrochar and graphene; Schematic diagram of digesters for anaerobic digestion of thin stillage with conductive materials amendment (Figure S1); The performance of (A) biomethane yield and (B) VFAs formation and COD concentration of the blank group during anaerobic digestion after acidic shock (Figure S2). Characteristics of thin stillage and inoculum (Table S1); Soluble COD concentrations of pyrochar and graphene (Table S2).

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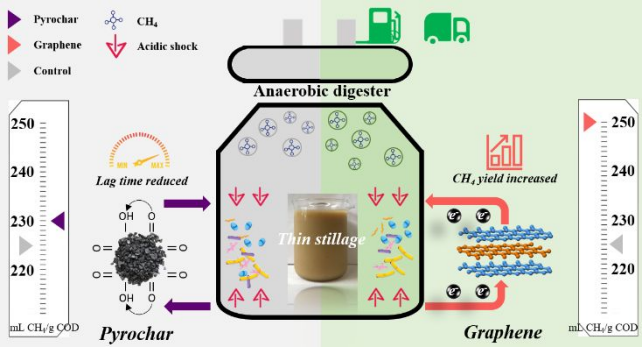
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Synopsis: Graphene can alleviate acidic shock and improve biomethane production during digestion of thin stillage.