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Anaerobic digestion has long been considered a sustainable means of waste stream management due to its capacity to valorize organic carbon content into biogas. However, the efficiency of the conversion process is often limited during the treatment of complex waste sources. In this work, we examine the combination of two emerging digestion enhancement approaches and their impacts on key microbial activity.

Increased applied voltage in the presence of GAC enhances microbial activity and methane production during anaerobic digestion of food waste

Moustapha Harb[1], Noel Ermer[2,3], Christelle BouNehme Sawaya[1], and Adam L. Smith*[,2]

[1] Department of Civil and Environmental Engineering, Lebanese American University, 309 Bassil Building, Byblos, Lebanon 2038-1401

[2] Astani Department of Civil and Environmental Engineering, University of Southern California, 3620 South Vermont Avenue, Los Angeles, CA, USA 90089

[3] Marymount High School, 10643 Sunset Boulevard, Los Angeles, CA, USA 90077

*Corresponding author:
Adam L. Smith
3620 South Vermont Avenue, Los Angeles, CA, USA 90089
Phone: 213-740-0473
Fax: 213-744-1426
Email: smithada@usc.edu

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Abstract

Suspended conductive materials and bioelectrochemical systems (BES) have independently been shown to improve methane production by enhancing anaerobic digestion (AD). Little is known, however, about their potential combined effect in AD systems. This study investigates a BES-AD system with and without granular activated carbon (GAC) supplementation under applied voltage ranges of 1.25V and higher. Results indicated that the BES-AD system with GAC could achieve robust methane production (>300 mL CH_4/g COD) from food waste at up to 2.75V, whereas the BES-AD system alone did not. Microbial analysis revealed that improved performance of the BES-AD system with GAC coincided with higher relative activity of exoelectrogenic bacteria (*Geobacter*) on anodic biofilms. This advantageous application of GAC also resulted in *Methanospirillum* emerging as a dominant contributor to methanogenic relative activity in cathodic and GAC biofilms. These observations suggest that suspended GAC can serve to enhance the already beneficial coupling of BES with AD.

1. Introduction

In light of the ever-increasing worldwide threats of climate change and population growth, an eminent need for new energy-positive waste valorization technologies is clear. Organic waste streams offer unique prospective opportunities in this regard, given that the anaerobic digestion (AD) of such wastes can serve a multifaceted purpose in environmental preservation and energy recovery. The possibility of upgrading AD-associated biogas methane content to levels near those of biomethane holds far reaching implications for future systems' integration with the gas

grid.[1] Moreover, the AD process' inherently low energy requirements have the potential to further reduce carbon footprints for wastewater treatment. A variety of waste streams are already commonly treated using anaerobic digesters, and food waste is specifically attractive in this regard due to it's potential for decentralized applications at high organic loading rates.[2]

Since the discovery of direct interspecies electron transfer (DIET) as a microbiological phenomenon, interest in its employment for practical engineered applications has been on the rise. Not surprisingly, a surge of studies investigating DIET mediated AD has followed in recent years, with the overall intent of improving process performance.[3] Much of this research has focused on the addition of various forms of conductive materials that include magnetite, graphite, biochar, granular activated carbon (GAC), and carbon cloth to enhance DIET in AD systems.[4] Almost all of the recent work employing conductive material as part of the AD process has found that digester performance and methane production were enhanced in their presence. Such studies have suggested that DIET is indeed responsible for improvements in methane generation through various high-throughput molecular techniques.[4]

Another means for the enhancement of AD is its combination with bioelectrochemical systems (BES). This area has also been a topic of great interest as of late, with a range of studies investigating the effect of AD-microbial electrolysis cell (MEC) integration.[5, 6] This combination of systems has also shown great promise, with a majority of studies indicating improvements in overall methane production, both in terms of total methane generated and in methane biogas percent. Results from BES-associated work have suggested that electrode-based applied voltages as low as 0.5V can improve methane production by affecting both bioelectrode and bulk solution microbial communities.[7] Given the high sludge density in typical anaerobic digesters, however, it is unclear to what extent the suspended anaerobic microbial consortium can be directly enhanced.[8] For example, one recent study has shown that applying potentials to

conductive materials during AD, although enriching for DIET-associated exoelectrogens and methanogens, actually caused a deterioration of performance at higher organic loading rates.[9]

Certain studies have focused on examining BES-AD systems at higher voltages than traditionally applied in MECs. Some such work has indicated that voltages of up to 2V can actually enhance total methane production and biogas content, despite an increased potential for failure due to electrolysis.[10, 11] Although higher voltage application concomitantly requires greater power input (reducing energy benefits of enhanced methane recovery), there remains the potential for co-application of such systems. For example, increasing applied voltages have been shown to significantly improve membrane-fouling rates in BES-coupled anaerobic membrane bioreactors (AnMBRs) while also improving methane generation.[12] Coincidentally, GAC has also been well-established as an effective means for membrane fouling mitigation in AnMBR systems (e.g., in anaerobic fluidized membrane bioreactors),[13] and their combination with BES systems has already been described as an imminent technology.[14] However, it remains to be determined how GAC addition might affect the performance of a BES-AD system above currently reported upper-limit applied voltages (which could be concurrently beneficial for membrane-based system integration).

Recent work has, indeed, revealed the effective advantages of combining BES-AD with conductive materials (magnetite) for improving system performance by promoting DIET.[15] Further, food waste, specifically, has been identified as a potentially ideal application of BES-AD systems.[16, 17] However, the complex and widespread nature of this waste source requires further development of innovative solutions for its treatment to achieve optimal biogas valorization. The incorporation of low-cost materials such as GAC in higher applied voltage BES-AD membrane-integrated systems may well be the new frontier of such solutions. However, due to the importance of electroactive bacteria and archaea in all of the above scenarios, investigation

of new applications must take into account the roles of these key microbial groups. Understanding the interactions between syntrophic exoelectrogens and methanogens remains an evolving area of research,[18, 19] with key microbial activity in mixed-community anaerobic digester environments being a specifically understudied area.[20] With this in mind, the work of the present study was conducted with two primary objectives: (1) to evaluate the effect of suspended GAC in combined BES-AD systems on methane production (at increasing applied voltages) and (2) to assess the microbial 16S rRNA relative activity of the suspended biomass, the electrode-attached biomass, and the GAC-attached biomass in these systems.

2. Materials and methods

2.1 Experimental set-up

To investigate the effects of combined BES-AD systems with GAC, four different lab-scale anaerobic reactor setups (0.5 L each) were run in triplicate. All reactors were operated in batch mode at a controlled temperature of 35°C. Reactor setups included: (R1) control anaerobic digesters (no applied voltage and no GAC), (R2) control anaerobic digesters with GAC (no applied voltage), (R3) anaerobic digesters with applied voltage (BES-AD, no GAC), and (R4) anaerobic digesters with applied voltage and GAC (BES-AD-GAC). Reactor setup and corresponding sample types are shown in Figure 1. Experiments were performed at applied voltages of 1.25V, 1.75V, 2.25V, and 2.75V under a fixed loading rate (per feeding cycle) of 10 g/L food waste COD, which resulted in an organic loading rate (OLR) of 1.67 g COD/L-d. Because all reactors were fed and monitored in triplicate, it was necessary to conduct the increasing applied voltage experiments at different times (sequential runs). In order to account for the

potential impact of temporal changes in both system performance and microbial communities, all control reactor replicates (R1) and non-BES GAC reactor replicates (R2) were evaluated in parallel with the BES-AD systems for all applied voltages tested (R3 and R4). A subsequent experiment under incremental food waste COD loading rates of 10, 15, and 20 g/L (OLRs of 1.67, 2.5, and 3.33 g COD/L-d, respectively) at a fixed applied voltage of 1.75V was also conducted.

Active anaerobic sludge was homogenized and added to each reactor at equal mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) concentrations of 6.5 and 7.5 g/L, respectively. Reactors were fed with 115 g COD/L food waste at the beginning of each feeding cycle (6 d). Food waste used in this study was obtained from Divert, Inc. (Compton, CA) and was homogenized prior to COD characterization and feeding. GAC-containing reactors were supplemented with 15 g of GAC per 0.5 L reactor (resulting in 30 g/L each) prior to commencement of the experiment. Physical properties of the GAC used included particle sizes of 2-5 mm, surface area of 1300-1400 m^2/g, and density of 1.8-2.1 g/cm^3 (Sigma-Aldrich, CAS 7440-44-0). All reactors were mixed continuously using magnetic stirrers operating at 120 rpm. BES-AD reactors were set up with graphite (anode) and stainless steel (cathode) electrodes that were connected to a programmable linear power supply, Model No. PPS2320A (Circuit Specialists, Tempe, AZ, USA) by titanium and stainless-steel wires for anode and cathode, respectively. Anodic surface area per reactor was 1900 mm^2 and cathodic surface area per reactor was 2700 mm^2. Average current density per cycle (A m^{-2}) was calculated based on cathodic surface area and the current (mA) measured across an in-line resistor. Feeding cycles ranged from 6-7 days, and were determined complete based on the time required for the cumulative biogas production curve to reach a 1/15 slope of that observed for the peak biogas production phase.

2.2 Performance characterization

Biogas production was monitored continuously from each reactor replicate by an on-line respirometer, Model No. BPA-800 (Challenge Technology, Springdale, AR, USA). Biogas was characterized for methane content by gas chromatography with flame ionization detection (GC-FID) three times per feeding cycle using a Trace 1310 GC system (Thermo Scientific, Waltham, MA, USA) based on methods described previously.[21] MLSS and MLVSS were measured in accordance with USEPA Method 160.4. Chemical oxygen demand (COD) was quantified in accordance with USEPA Method 410.4 using a spectrophotometer, Model No. HI801 (Hanna Instruments, Woonsocket, RI, USA). Volatile fatty acids (acetate, propionate, and butyrate) were measured using ion chromatography on an ICS 2100 (Thermo Fisher Scientific, Waltham, MA, USA) based on methods described previously.[21]

2.3 Biomass sampling and microbial community characterization

Biomass samples were taken for suspended sludge, GAC-attached biofilm, cathode surface biofilm, and anode surface biofilm for representative replicates of each reactor system (one reactor was sampled from each set of replicates), depending on sample type presence in each system. Biomass was sampled for all four reactor types (R1-R4) under the incremental voltage phase of the experiment for applied voltage feeding cycles of 1.25V, 1.75V, and 2.25V. For R1-R4, 2 mL was taken per sample of suspended sludge, centrifuged at 5,000 x g for 10 min at 4°C and immediately stored at -80°C after supernatant decanting. GAC biofilm was sampled for reactors R2 and R4 by recovering 60-90 mg of biofilm-GAC from each reactor per sampling event

and storing at -80°C. Anode and cathode biofilm samples were taken for reactors R3 and R4 by scraping a sufficient mass (30-70 mg) of biofilm from the electrode surfaces using a sterile spatula and stored at -80°C. For all of the above described sampling procedures, biomass to be extracted for RNA was suspended in 1.5 mL of DNA/RNA Shield (Zymo Research, Irvine, CA, USA) prior to storing at -80°C.

RNA extractions were performed on the stored samples using the Maxwell 16 simplyRNA Blood kit (Promega, Madison, WI, USA) according to the manufacturer's protocol as described previously.[22] Extracted RNA concentrations were determined using a BioSpectrometer (Eppendorf, Hamburg, Germany). Single-stranded complementary DNA (cDNA) was synthesized from RNA extracts by reverse transcription using the SuperScript VILO cDNA Synthesis Kit according to the manufacturer's protocol (Life Technologies, Grand Island, NY, USA). Synthesized cDNA (RNA) from each sample was subjected to PCR to amplify the 16S rRNA gene using a universal 16S rRNA gene primer set targeting the V4 region.[23] PCR reactions consisted of a total volume of 20 µL that included 100 nM each of the forward and reverse primers, 17 µL AccuPrime Pfx SuperMix (Invitrogen, Carlsbad, CA, USA), 0.5 ng template, and nuclease-free water. Thermocycling for amplification consisted of 2 min denaturation at 95°C, 30 cycles of denaturing at 95°C for 20 s, annealing at 55°C for 15 s, and extension at 72°C for 5 min, and final extension at 72°C for 5 min. Amplicons were pooled by equal mass using the SequalPrepTM Normalization Plate Kit (Life Technologies, Grand Island, NY, USA). Multiplexed amplicons were sequenced on the MiSeq platform (Illumina, San Diego, CA, USA) using the MiSeq Reagent Kit V2 by the Microbial Systems Molecular Biology Laboratory (University of Michigan, Ann Arbor, MI, USA).

FASTQ files attained from high-throughput sequencing were analyzed using the mothur bioinformatics platform based on the Schloss MiSeq SOP.[23, 24] The UCHIME algorithm was used

to filter sequences for quality and remove chimera to generate high-quality reads. The SILVA reference database was used to align filtered sequences.[25] Operational taxonomic unit (OTU)-based clustering was performed based on an average neighbor algorithm at a 3% cutoff limit. Subsampling of clustered sequence files was performed to normalize sequences per sample based on the output file with smallest number of total sequences. Taxonomical classification of sequences was done using the Ribosomal Database Project (RDP) Classifier database against the 16S rRNA gene Training Set Version 15[26] and using the BLASTN algorithm on the National Center for Biotechnology Information (NCBI) database.[27] Per-sample normalized results for sequencing of 16S rRNA (after conversion of amplified rRNA to cDNA) are subsequently referred to as relative activity.

3. Results and discussion

3.1 BES-AD with GAC enhanced total methane generation

Experiments were conducted with all reactor types operating in triplicate for each feeding cycle to minimize any misinterpretation of results arising from temporal changes in biomass activity. Overall, methane generation rates indicated that AD systems with only GAC (R2), AD systems with only applied voltage (R3), and AD systems with both BES and GAC addition (R4) all increased total methane production compared to control AD systems (R1) during incremental applied voltage cycles of 1.25V and 1.75V at 10 g/L COD organic loading (Table 1). At 2.25V, however, both R3 and R4 showed significant improvement in methane production in

comparison to R1 and R2, indicating a potential advantage of BES-AD systems for improving COD-methane conversion efficiencies at higher applied voltages (> 90%). Upon increasing applied voltages to 2.75V, methane production in R3 ceased nearly immediately in all reactor replicates. During the same applied voltage feeding cycle, though, overall methane production in R4 was maintained in all three replicate reactors, although with greater variability than was observed for the same reactors in previous cycles (345 ± 89 mL CH_4/g COD). This strongly suggests that the presence of GAC was a key factor in maintaining anaerobic biomass activity in BES-AD reactors at applied voltages that would otherwise inhibit methanogenesis due to electrolysis of water. In a follow-up experimental phase, the effect of increasing organic loading rates at a fixed applied voltage (1.75V) was also investigated. Results of this phase indicated that combined BES-AD with GAC (R4) was also beneficial for maintaining methane production rates at organic loading rates of up to 20 g COD/L.

Baseline methane production from food waste digestion in R1 during the fixed organic loading experiment phase was generally consistent for each incremental feeding cycle (1.25V-2.75V) at 242-253 mL CH_4/g COD. In R2, average methane production was consistently higher than R1 at 274-316 mL CH_4/g COD (P = 0.011, unpaired t-test) for all incremental voltage feeding cycles (1.25V-2.75V). Given that no actual BES system was present in either R1 or R2 over the four sequential cycles, these results indicate that the presence of GAC at 30 g/L consistently improved overall methane volumes from these batch AD systems. This observation is in line with those of previous studies aimed at evaluating GAC presence during AD, all of which showed that the presence of this conductive material served to enhance total methane generation.[28-30] These mixed-community studies have been inconclusive in identifying whether this enhanced reactor performance is a result of increased direct interspecies electron transfer (DIET) or improved syntrophic interactions during methanogenesis. However, in at least two cases, increased

Geobacter and hydrogenotrophic methanogen relative abundances were also seen in the presence of GAC.

Both BES-combined reactor types (R3 and R4) yielded similar methane generation rates and methane biogas content to those of the GAC-only system (R2) at applied voltages of 1.25V and 1.75V (Table 1). However, at an applied voltage of 2.25V, both reactors R3 and R4 resulted in much-improved methane production. Specifically, total methane production in R3 and R4 was 345 ± 29 and 363 ± 12 mL CH_4/g COD, respectively. These methane generation rates indicate near complete methane conversion at 90% and 95% of maximum theoretically producible values, respectively (at biogas recording temperature of 25 °C). These COD-normalized methane production rates were partially attributable to improved average biogas methane content, which reached as high as 79% in R4 at 2.25V. A comparison of average increases in MLVSS of the different reactor types indicated that the higher methane production rates in both BES-AD systems as compared to the non-BES controls was in parallel with lower overall biomass accumulation in the reactors. Average increase in MLVSS per cycle for reactor types R1-R4 was determined to be 0.85 ± 0.05, 0.32 ± 0.11, 0.05 ± 0.01, and -0.07 ± 0.02 g MLVSS/L, respectively. These results suggest that BES-AD systems can be beneficial for the valorization of complex waste streams in comparison to AD alone.

The improved methane production and biogas content of R3 and R4 at applied voltages of over 2V are not the first time such elevated voltages were observed to improve AD-BES system performance. A recent study conducted in a BES-AD experiment at a similar range (up to 2.0V) also indicated heighted COD-methane conversion efficiencies and maximum methane content at increased voltage rates.[10] This implies that in combined BES-AD systems, significantly higher applied voltages than those considered ideal in MECs may be beneficial to the methanogenic process. Such observations may be attributable to the inherently lower

coulombic efficiencies of BES-AD systems as compared to MEC systems alone. A recent study investigating this phenomenon found that the enrichment of electroactive microorganisms in the bulk solution of a bioelectrochemical anaerobic digester and not the electrode biofilm-dependent coulombic efficiencies were primarily responsible for methane production in such systems.[7] Our results showed that differences in coulombic efficiency between R3 and R4 may have also contributed to the overall higher methane generation of the BES-AD system with GAC (R4). This was reflected in the higher measured average current density per cycle of R4 as compared to R3 at 4.22 ± 0.38 and 7.56 ± 0.49 A m^{-2}, respectively. In addition to increasing overall methane generation in R4 at 2.25V, this improved BES efficiency was likely also a contributing factor in supporting the BES-AD with GAC system's resistance to deterioration during the 2.75V feeding cycle.

3.2 Sample type dictated the predominantly active microbial populations

Microbial communities of anaerobic digester biomass are known to have highly variable gene expression levels in comparison to actual bacterial presence as characterized by 16S rRNA gene abundance.[31] With this in mind, the present study aimed to characterize the 16S rRNA relative activity of the microbial communities in biomass samples rather than 16S rRNA gene relative abundance. Although 16S rRNA analysis alone is not directly indicative of relevant functional or fully-normalized gene expression, it allows for representation of the total microbial community due to its universality among both bacteria and archaea. Overall, results were indicative of the vastly different relative activities expected to be present on the different biomass sample types. Representative results comparing anodic, cathodic, suspended sludge, and GAC biomass in the

BES-AD system with GAC (which contained all sample types) operated at 1.25V are shown for dominant bacterial and archaeal groups (> 1% average relative abundance) at the genus-level in Figures 2A and 2B, respectively.

Suspended sludge biomass across all four reactor types (R1-R4) was widely dominated by the genus cluster *Clostridium sensu strico*, with relative activities ranging from 18-28%. Representative identified OTUs indicated that this group was most closely related to the species *Clostridium tertium* (99% similarity), which is a facultative anaerobe that has been previously found as a key microbial group in anaerobic digesters treating food waste.[32] The broad dominance of relative activities by *Clostridium sensu strico* across all reactors' suspended sludge is indicative of its essential role in the anaerobic process, although a lack of variability suggests that it was not limiting under the various tested conditions. Unclassified Anaerolineaceae was also consistently detected in suspended sludge at relative activities of over 5%. This may be indicative of groups from this family playing a key role in treatment of food waste, especially considering their previously observed advantages for the anaerobic treatment of complex organic substrates.[33] Other primary groups classified at the genus-level in suspended sludge samples included *Pelotomaculum*, *Trichococcus*, and *Lactobacillus*, which consistently made up over 1% relative activity of the suspended biomass. The main methanogenic relative activity in the suspended biomass included *Methanobacterium* (9-18%), *Methanosarcina* (5-9%), and *Methanothrix* (4-8%), indicating a general balance of potential methanogenic pathways.

Cathode and GAC biomass samples were generally similar in relative activity to those of the suspended sludge samples (Figures 2A and 2B). Exceptions to this were primarily among methanogenic genera. *Methanoculleus* had a more robust presence in GAC biomass (3-11%) as compared to suspended, indicating a shift in the dominant hydrogenotrophic methanogenic groups. Nonetheless, *Methanobacterium* maintained its relative activity in cathode biofilms (11-

27%), despite comparatively higher levels of *Methanospirillum* and *Methanoculleus*. The dominance of *Methanobacterium* in cathodic biofilms is an indication that a major fraction of methane produced through BES-assisted methanogenesis was the result of hydrogen evolution from the cathode surface, as they are not known to be capable of direct electron uptake.[19]

Anodic biomass relative activity was highly dissimilar to the other three biomass types mentioned above. *Geobacter* was the dominant genus in anodic biofilms, comprising up to 70% of total anodic relative activity, which is also in agreement with previous observations in BES-AD systems.[5] Representative identified OTUs associated with *Geobacter* indicated that a majority of its relative activity was likely attributable to *Geobacter anodireducens* (100% similarity). *G. anodireducens* is a novel exoelectrogenic species that was isolated from a bioelectrochemical system and is capable of tolerating higher salinity levels than other commonly studied *Geobacter* species.[34] Considering that a majority of BES work is typically performed using simple substrates under controlled conditions, it is possible that a factor in its selective enrichment in the current study's anode biomass is an indication of its robustness in such systems for the treatment of food waste. Other microbial groups that made up significant relative activity in the anode biomass included unclassified Porphyromonadaceae and *Lactobacillus*. Although *Lactobacillus* was not active in anode biofilms at 1.25V (as shown in Figure 2A), its contribution to relative activity in the anode biomass increased significantly at higher applied voltages (discussed in Section 3.4). This is a potentially significant observation considering that species of this genus have previously been used as anode respiring bacteria in BES systems.[35] *Lactobacillus* is a bacterium that is known to be selectively enriched during food waste digestion, which may have also played a role in its increased relative activity.[36]

3.3 Higher activity of *Methanospirillum* observed in GAC biomass of BES-AD system

The GAC biomass of both R2 (AD with GAC) and R4 (BES-AD with GAC) showed consistently higher relative activity of *Methanoculleus* as compared to corresponding suspended sludge biomasses, with concurrently lower relative activity of *Methanobacterium* (Section 3.2). To elucidate the differences between the GAC biomasses, relative activities were also compared for feeding cycles of R4 at 1.25V, 1.75V, and 2.25V, along with the parallel feeding cycles for R2. Results indicated that bacterial relative activities were generally not significantly different. One exception was in the case of *Geobacter*, which was higher in R4 GAC biomass at 3.01 ± 1.61 % average relative activity as compared to R2 at 0.31 ± 0.28 % (P = 0.046, unpaired t-test). OTU-based analysis showed that *Geobacter*-associated groups in the GAC biomass were also related to *G. anodireducens*.

Analysis of methanogenic activity based on dominant OTUs revealed that groups most closely related to *Methanobacterium aarhusense* (97% similarity) were slightly lower in R4 GAC biomass relative activity as compared to R2 (Figure 3), but not at statistically significant ranges (P = 0.129, unpaired t-test). Further, although it was determined that OTUs associated with *Methanoculleus receptaculi* (99.5% similarity) and *Methanosarcina soligelidi* (98% similarity) showed substantial relative activity in GAC biomass of both R2 and R4, no noteworthy differences were observed between the two reactors. Perhaps most importantly, it was determined that multiple OTUs associated with *Methanospirillum hungatei* (> 95% similarity) exhibited significantly higher relative activity in R4 GAC biomass as compared to that of R2 (Figure 3, P = 0.047, unpaired t-test). This observation has far-reaching implications, considering

that *M. hungatei* has been recently determined to possess highly unique electrically-conductive protein filaments.[37] These filaments have the potential to serve as long-range electron transfer conduits between microbes. Based on this distinctive extracellular capacity of *M. hungatei*, it is likely that heightened activity of this microbial group would play a key role in mediating electron transfer between conductive GAC granules and the bulk suspended sludge solution/electrode biomass (based on surface scouring during continuous mixing). This phenomenon could, indeed, be partially responsible for the higher overall methane production rates by the BES-AD with GAC system (R4) as compared to other reactor types at 2.25V, especially considering that *M. hungatei* showed maximal relative activity (> 9%) at this applied voltage. Further, it also seems plausible that the presence of this species would have aided in the dissipation of electrons into and through the bulk solution, thereby aiding in R4's comparative robustness at the highest applied voltage (2.75V).

3.4 Electrode biomass microorganisms influenced by presence of GAC

Analysis of relative activities of both the anodic and cathodic biofilms revealed potentially important differences in key microbial groups on the respective electrodes of R3 and R4. Further, drastic shifts in anode-respiring bacteria were observed in the anode biomass at increasing applied voltages for both R3 and R4. These differences were indicative of a distinct advantage for the BES-AD with GAC system (R4) as compared to the BES-AD system alone (R3) from a microbial activity perspective.

Geobacter activity was consistently higher in R4 anodic biomass as compared to R3, with relative activities ranging from 10-69% and 0.5-38%, respectively (Figures 4A and 4B). Still, this observation was coupled with a marked decrease in *Geobacter* relative activity at incremental applied voltages of 1.25V, 1.75V, and 2.25V in both reactors R3 and R4. Conversely, *Lactobacillus* relative activity increased at these incremental applied voltages, indicating a shift in the predominant anode-associated groups. It is noteworthy, however, that *Geobacter* and *Lactobacillus* combined to makeup over 20% of total microbial relative activity in R4 at 2.25V, whereas in R3 at 2.25V they made up less than 6% (with *Geobacter* making up only 0.5%). The predominant *Lactobacillus* OTU identified in these samples was most closely related to *Lactobacillus fermentum* (100% similarity), which is known to form strong biofilms and possess cell walls that resist lysis.[38, 39] This may have been an advantage for their selective increase in relative activity during heightened applied voltage exposure, as such voltages have been shown to lead to anodic bacterial cell lysis in MEC systems.[40] Further, their co-activity with *G. anodireducens* in R4 could also have been a key factor in maintaining biogas production at higher applied voltage levels.

Analysis of the cathodic biomass from a methanogenic perspective revealed that a majority of the dominant genera on the cathode were not significantly different between R3 and R4 reactor samples (Figures 4C and 4D). Further, no apparent trends were observed for any methanogens in correspondence with increasing applied voltage during subsequent feeding cycles. In fact, the only substantial difference that was observed between R3 and R4 cathodic biomasses was the significantly higher relative activity of the genus *Methanospirillum* in R4 samples (P = 0.003, unpaired t-test), which was again predominated by an OTU related to *M. hungatei*. This finding reiterates the potential importance of *M. hungatei*'s role in facilitating long-range electron transfer in biofilm biomasses of the BES-AD with GAC system. The

heightened relative activity of *M. hungatei* in both cathodic and GAC biomass could have concurrently aided in the dissipation of electrons taken up from the cathode while improving the overall conductivity of the AD-GAC fraction of the system. Although it was not possible to prove as part of the current study, this phenomenon may well have been a key factor that resulted in the observed overall R4 system robustness and methane conversion efficiency.

4. Conclusions

This study aimed to evaluate the effect of combining suspended GAC with a BES-assisted AD system. Results indicated that such a system could indeed increase methanogenic viability at high applied voltage levels as compared to operation in the absence of suspended GAC. Analysis of microbial relative activity in different biomass sample types revealed that *Methanospirillum* may play a key role in facilitating electron transfer within the cathodic and GAC microbiomes. These findings suggest that the previously confirmed advantageous enhancement of anaerobic digestion using BES and suspended conductive materials (separately) can potentially be improved upon through their combined application.

5. Conflict of interest

The authors declare no conflicts of interest.

6. Acknowledgements

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7. Supplementary information

Supplementary information of this work can be found in the online version of the paper. The supplementary data shows methane generation and biogas methane content during increasing food waste OLR experiments for all reactor types and average and maximum current densities measured for increasing applied voltages in reactors R3 and R4 during the primary experiment.

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Tables

Table 1. Total methane produced (in mL CH_4/ g COD) from all anaerobic digester categories and measured biogas methane content percentage (shown in parentheses) from each reactor at incremental voltages of 1.25V, 1.75V, 2.25V, and 2.75V. Reactors R1 and R2 were operated as parallel control experiments (no applied voltage).

	1.25V	**1.75V**	**2.25V**	**2.75V**
R1 (Control)	247±54 (67±4)	242±56 (66±2)	253±59 (68±2)	247±50 (65±1)
R2 (GAC only)	305±27 (73±2)	316±60 (70±1)	274±58 (67±2)	287±52 (66±1)
R3 (BES only)	315±21 (69±1)	314±19 (67±2)	345±29 (73±1)	13±3 (2±2)
R4 (BES with GAC)	294±41 (70±4)	311±16 (70±3)	363±12 (79±6)	345±89 (64±15)

Figures

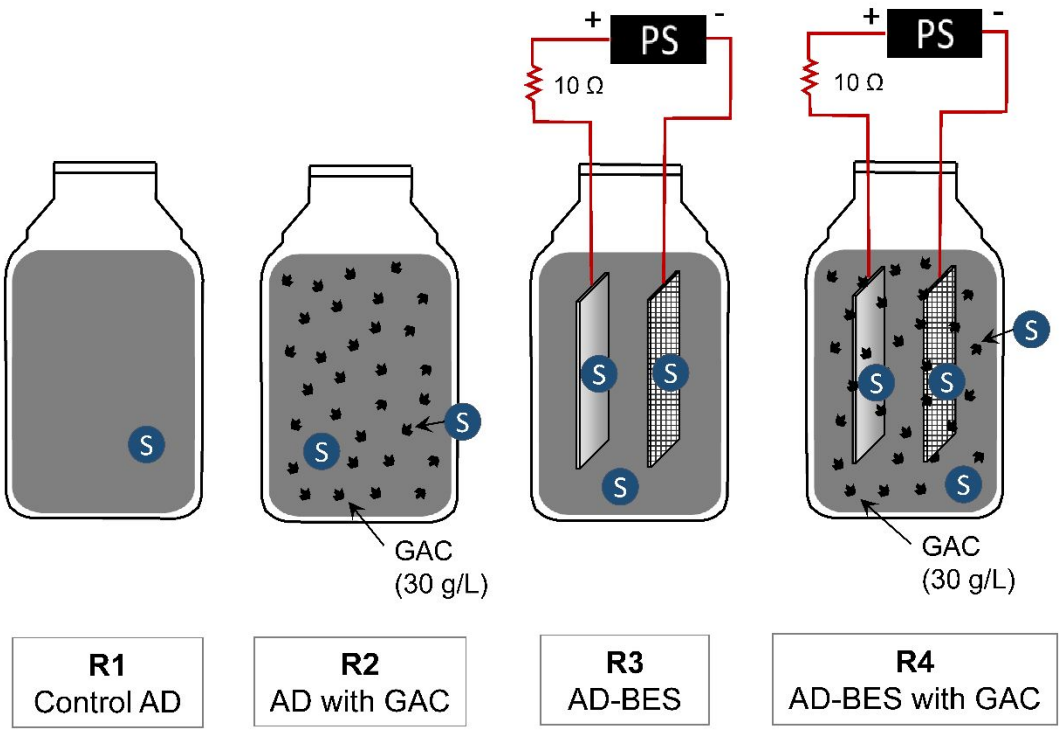


Figure 1 Schematic representation of the lab-scale reactor setups used in this study. R1 represents a control completely mixed anaerobic digester with only suspended sludge, R2 represents an anaerobic digester with 30 g/L suspended GAC but no bioelectrochemical system (BES), R3 represents an anaerobic digester operating with BES but no GAC, and R4 represents the combined anaerobic digester system with both BES and GAC. Circles with an "S" indicate the different sample types obtained from each reactor system.

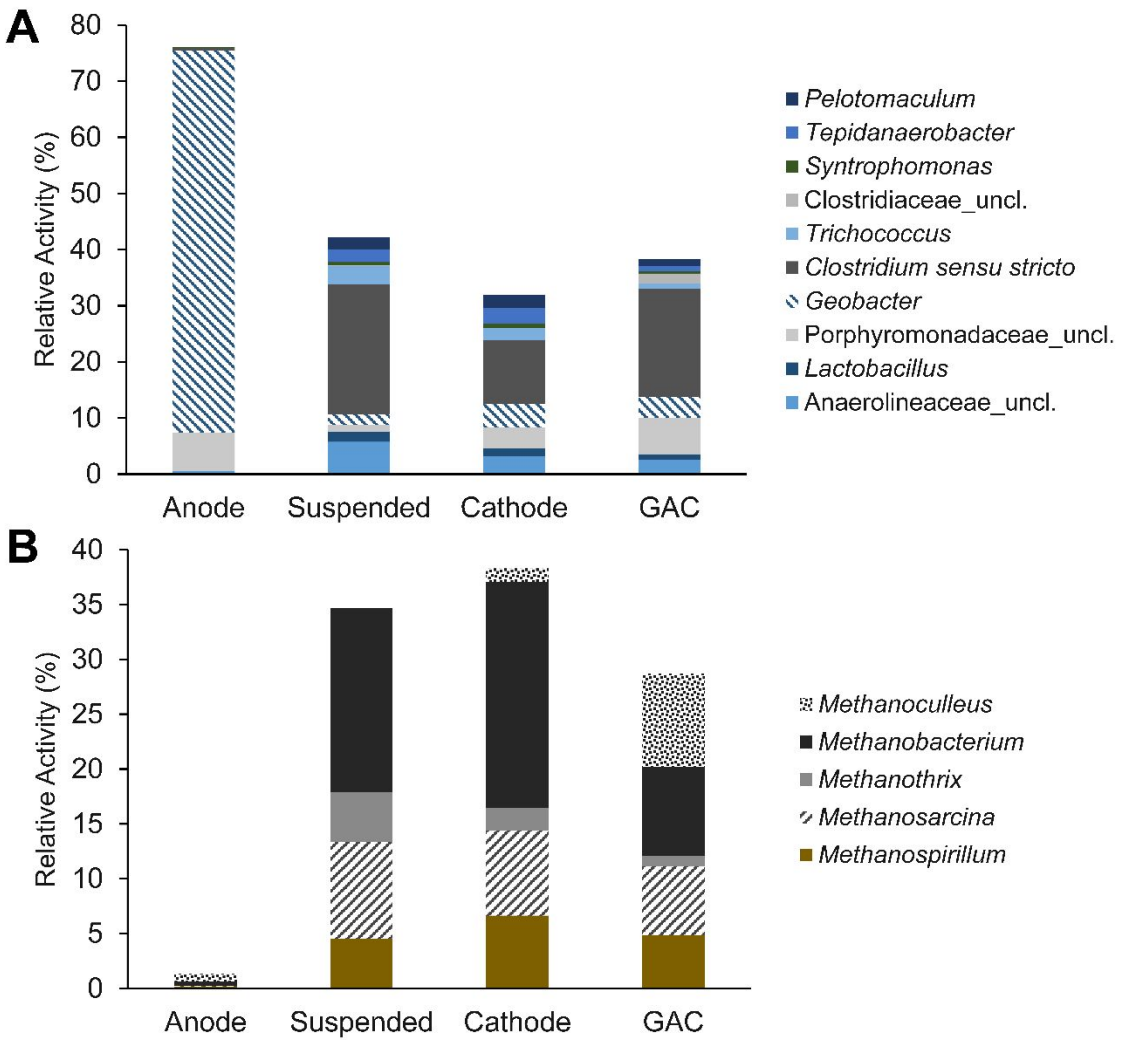


Figure 2 Relative activities (%) of the dominant microbial groups classified to the genus or family-level for **(A)** Bacteria and **(B)** methanogens in the different sample types of the BES digester system with GAC (R4) when operated at 1.25V applied voltage. The four sample types represented in the R4 system were anode biofilm, suspended biomass, cathode biofilm, and GAC biofilm.

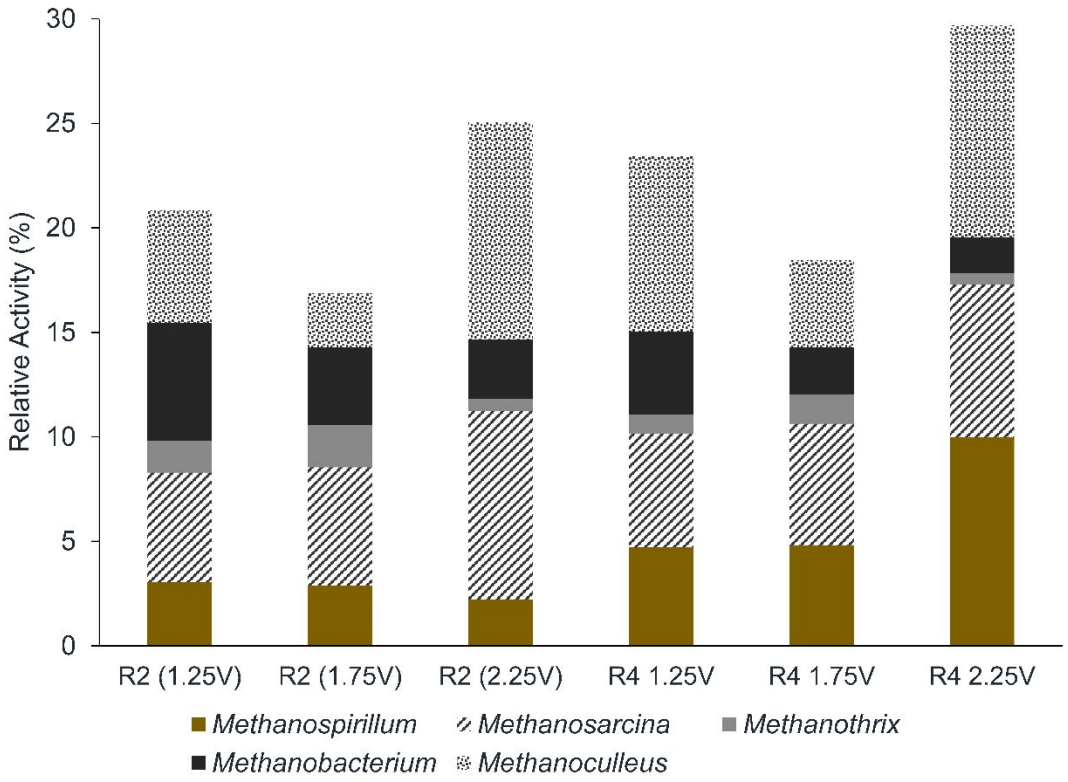


Figure 3 Relative activities (%) of the dominant methanogenic genera observed in GAC biofilms from both GAC-containing anaerobic digesters (R2) and GAC-containing BES-based anaerobic digesters (R4). Parallel runs were sampled for both R2 and R4 during the increase in applied voltage from 1.25V to 2.25V in reactor R4. Genera represented here were identified from the top ten operational taxonomic units (OTUs) shown to have highest similarity to methanogenic archaeal species at a 16S rRNA sequence match of 95% or greater.

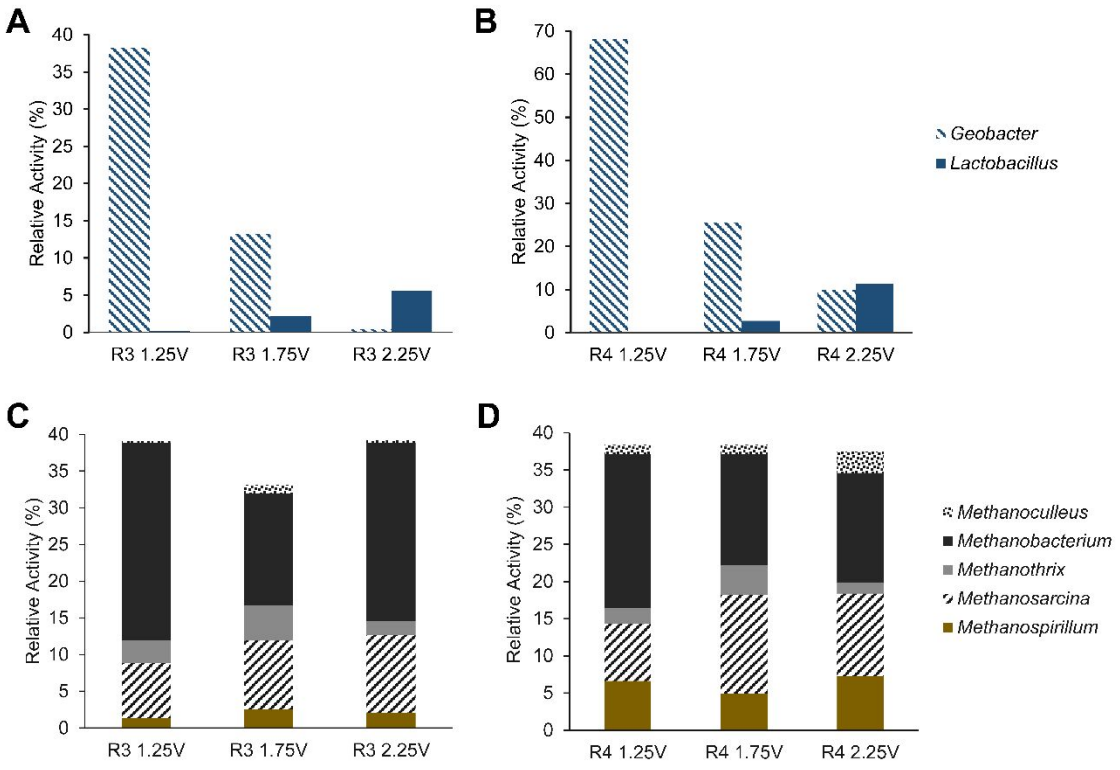
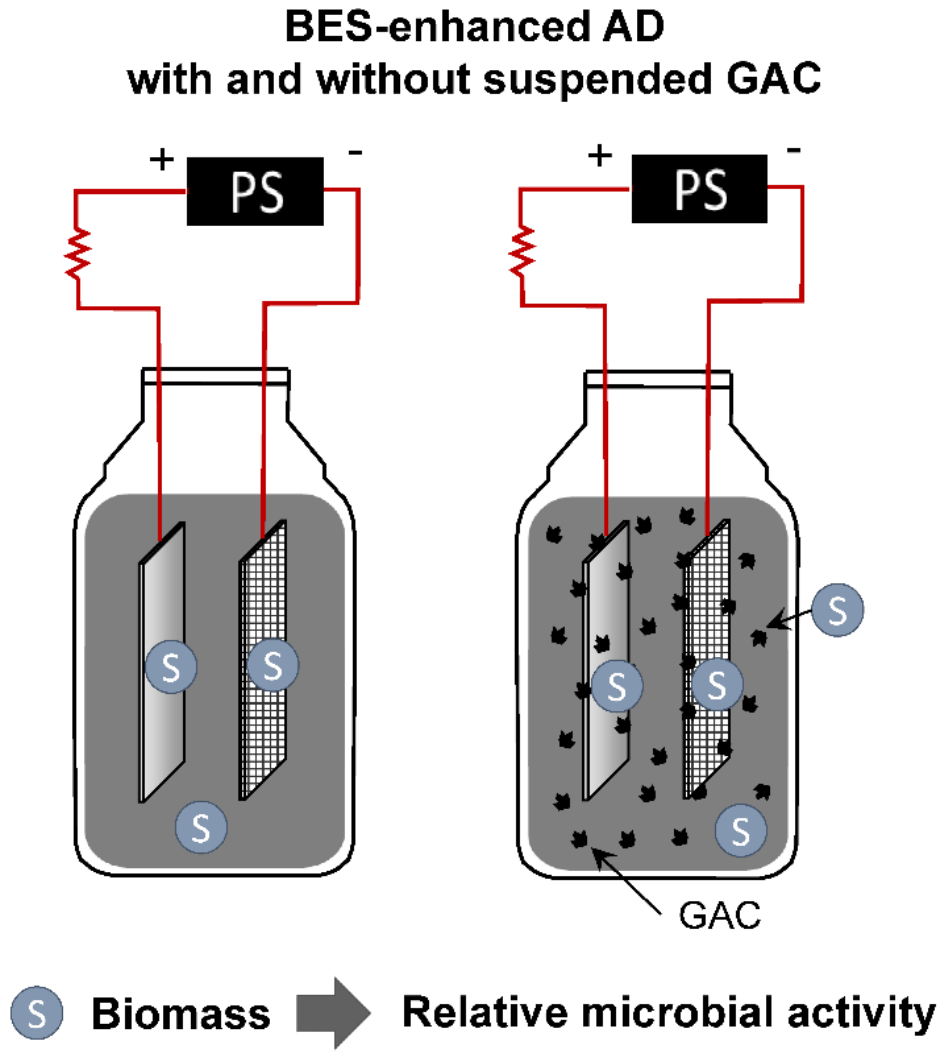


Figure 4 Relative activities of **(A)** dominant genera associated with exoelectrogenic species in anode biofilms of the BES anaerobic digester (R3) at varying applied voltages, **(B)** dominant genera associated with exoelectrogenic species in anode biofilms of the BES anaerobic digester with GAC (R4), **(C)** methanogenic genera in cathode biofilms of R3, and **(D)** methanogenic genera in cathode biofilms of R4.



1

2

3 Assessment of key microbial activities during the combined bioelectrochemical
4 and conductive material-based enhancement of anaerobic digestion.