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Zero valent iron-assisted methane production from anaerobic digestion of microalgae *Tetradesmus obliquus*

Seo-Yeon Yang¹, Won-Seok Kim^{2,3}, Se-Hoon Kim³, Jiyoung Choi⁴, Ji-Hoon Kim⁴, Michael J. Sadowsky^{5*} and Ji-Hoon Lee^{1,6*} 

Abstract

Anaerobic digestion (AD) of microalgae presents a promising strategy for renewable methane production while contributing to atmospheric CO₂ reduction. Methane production potential from two strains of the microalga *Tetradesmus obliquus* (AG60 and AG10) was investigated. To enhance biomass hydrolysis, zero-valent iron (ZVI, Fe⁰) was added as an in-situ facilitator. Methane yields from microalgae treatments significantly exceeded those of the control (accumulative methane of 93.2 mL/g volatile solid (VS) from seed sludge only), with AG60 and AG10 producing 139.7 mL/g VS (algae only, $p=5.0 \times 10^{-5}$) and 261.7 mL/g VS (algae only, $p=2.0 \times 10^{-6}$), respectively. Quantitative PCR analyses done targeting 16 S rRNA and the methanogen-specific *mcrA* genes revealed an increased abundance of methanogens. Microbial community analysis supported the increase in hydrogenotrophic taxa such as *Methanobacterium*, suggesting a shift toward hydrogenotrophic methanogenesis in the AD. This analysis also showed an increase in the relative abundance of cellulolytic bacteria, supporting more efficient degradation of algal biomass. Despite complex microbial interactions, the addition of microalgae resulted in a stable and functionally enriched microbial community that facilitated efficient methane production. These results demonstrate the feasibility of using microalgal biomass as a viable substrate for AD without extensive pretreatment and highlight its potential role in the development of sustainable biogas systems and climate change mitigation strategies.

Keywords Anaerobic digestion, Microalgae biomass, Methane production, Hydrogenotrophic methanogenesis, Microbial community dynamics

*Correspondence:

Michael J. Sadowsky
sadowsky@umn.edu
Ji-Hoon Lee
jhlee2@jbnu.ac.kr

¹Department of Agricultural Chemistry, Graduate School, Jeonbuk National University, Jeonju, Republic of Korea

²Division of Advanced Nuclear Engineering, Pohang University of Science and Technology (POSTECH), Pohang, Republic of Korea

³NC Square, Pohang, Republic of Korea

⁴Marine Geology & Energy Division, Korea Institute of Geoscience and Mineral Resources (KIGAM), Daejeon, Republic of Korea

⁵Department of Medicine, and BioTechnology Institute, University of Minnesota, Minneapolis, MN, USA

⁶Department of Bioenvironmental Chemistry, Jeonbuk National University, Jeonju, Republic of Korea

Introduction

The increasing global demand for renewable energy has intensified interest in anaerobic digestion (AD) as a sustainable technology for converting organic biomass into bioenergy including methane. AD is a microbially mediated process consisting of sequential hydrolysis, acidogenesis, acetogenesis, and methanogenesis, in which complex organic substrates are decomposed into methane and carbon dioxide by syntrophic bacterial and archaeal consortia [1, 2]. This technology has been widely implemented in wastewater treatment facilities, agricultural manure management, and food processing industries [3–6], owing to its ability to recover energy while simultaneously reducing waste volume and generating nutrient-rich digestate for agricultural reuse [7].

Among potential AD feedstocks, microalgal biomass has attracted considerable attention because of its rapid growth rate, high photosynthetic efficiency, and capacity for atmospheric CO₂ sequestration. In particular, green microalgae offer advantages such as high protein productivity and adaptability to wastewater-based cultivation systems [8, 9], in addition to a dual benefits of mitigating climate change and serving as a renewable energy source [10]. However, despite these benefits, methane yields from microalgal AD often remain lower than those obtained from conventional organic substrates. This limitation is primarily attributed to the recalcitrant nature of microalgal cell walls, which are frequently reinforced with non-hydrolyzable biopolymers such as algaenan, and to unfavorable substrate characteristics such as high nitrogen content and low biodegradability. Consequently, energy-intensive pretreatment methods (thermal, chemical, or enzymatic) are commonly required to enhance biomass accessibility, which undermines the net energy balance and economic feasibility of microalgae-based AD [11, 12]. A large number of researches examining biogas production from microalgal biomass have revealed that microalgae provide challenging substrates for AD, due in large part to their high cell wall resistance and high protein content [12], which is unsuitable for degradation conditions. To overcome these problems, additional pretreatment and co-digestion strategies are necessary for sufficient fermentation of microalgae biomass. For sustainable fuel production, however, cost/energy-intensive biomass pretreatment must be avoided to achieve a net positive energy process balance. Moreover, the complexity of microbial communities involved in AD necessitates a stable consortia of microorganisms to maintain optimal operational conditions. Key microbial groups in the methanogenesis pathway include hydrolytic bacteria, acetogens, and methanogens [1]. Therefore, microbial community composition and stability are critical factors in the performance and efficiency of AD systems.

Previous studies have demonstrated that zero-valent iron (ZVI) can enhance methane production by stimulating hydrogenotrophic methanogenesis, improving electron transfer efficiency, and modifying reactor redox conditions [13]. ZVI corrosion generates hydrogen and ferrous ions, which may serve as electron donors and promote syntrophic interactions among fermentative bacteria and methanogens. Furthermore, emerging evidence suggests that ZVI can facilitate direct interspecies electron transfer (DIET), thereby accelerating metabolic cooperation within AD microbial communities. Although these mechanisms indicate the potential of ZVI as a low-cost and in situ enhancement strategy, its application has largely focused on conventional substrates or a limited number of model microalgal species. This likely occurs by enhancing electron transfer among syntrophic microorganisms, particularly acetate-dependent methanogens, and thereby increasing methane yield [14]. Chlorophycean microalgae, such as *Tetradesmus obliquus*, possess cell walls reinforced with non-hydrolyzable biopolymers, creating a formidable barrier to bacterial hydrolysis and often resulting in sub-optimal methane yields. To overcome this recalcitrance without energy-intensive pretreatment, ZVI was applied as an in-situ electron donor and/or facilitator. ZVI is hypothesized to facilitate biomass degradation through abiotic reduction of cell wall polymers and modulation of oxidation-reduction potential (ORP), creating favorable conditions for methanogenesis.

A critical knowledge gap remains regarding the combined application of ZVI and structurally recalcitrant microalgal species that are underrepresented in AD research. Specifically, the effects of ZVI-assisted digestion on microbial community dynamics, methanogenic pathway selection, and methane yield enhancement in non-model microalgae have not been systematically investigated. Addressing this gap is essential for evaluating the broader applicability of ZVI-assisted AD and for improving the scalability of microalgae-based bioenergy systems. In this study, *Tetradesmus obliquus* was selected as the target microalgal strain due to its industrial relevance, robust growth performance, and notably resistant cell wall structure enriched with algaenan. These characteristics make *Tetradesmus* a representative yet challenging substrate for AD and an ideal model for evaluating strategies aimed at overcoming biomass recalcitrance. Rather than applying energy-intensive pretreatment methods, ZVI was introduced as a multifunctional in-situ facilitator to promote reductive reactions, enhance hydrolytic accessibility, and create favorable redox conditions for methanogenic activity. We hypothesized that (i) ZVI addition would improve methane yield by accelerating biomass degradation and electron transfer processes, (ii) ZVI would facilitate hydrogenotrophic methanogens,

particularly *Methanobacterium*, and (iii) ZVI-assisted digestion would result in a more stable and functionally robust microbial consortium. Accordingly, the specific objectives of this study were to quantitatively evaluate methane production performance from *Tetrademus obliquus* under ZVI-assisted anaerobic digestion conditions, to assess changes in microbial community composition and methanogenic functional gene abundance using molecular analyses, and to elucidate potential mechanistic links between ZVI-induced physicochemical changes and microbial metabolic responses. Through this integrated approach, this work aims to provide mechanistic insight into ZVI-assisted microalgal AD and to establish a scalable strategy for improving methane production efficiency from recalcitrant algal biomass.

Materials and methods

Incubation of microalgae

Five strains of microalgae were obtained from the Korean Collection for Type Cultures (Jeongeup, Republic of Korea). Preliminary growth studies indicated that two alga strains, *Tetrademus obliquus* (synonym: *Scenedesmus obliquus*) AG10047 and *Tetrademus obliquus* AG60783, showed relatively fast growth under high CO₂ conditions and were selected for further study. The strains are referred to AG10 and AG60, respectively. The microalgae were grown at 23–25°C under a 12-h photoperiod in Cyanobacteria BG-11 Freshwater Solution (C3061, Sigma-Aldrich, St. Louis, MO, USA). Incubations occurred under the mixed LED lights (white, red at approx. 660 nm, and blue at approx. 450 nm; with photosynthetic photon flux density of approximately 300–600 μmol m⁻² s⁻¹), with air supplied by a bubble generator.

Anaerobic digestion of microalgae

Seed sludge, obtained from the anaerobic digestion reactor of the municipal wastewater treatment facility operated by Jeonju Environment Office (Jeonju, Republic of Korea), was used as the source of microbiota. Total solids (TS) and volatile solids (VS) in the seed sludge and the two selected microalgae were measured according to the Standard Methods for the Examination of Water and Wastewater [15] and the results are presented in Table 1.

Anaerobic incubations were performed in batch conditions by using serum bottles with average total volume of 156.8 ± 0.8 mL. Prior to addition, the microalgal cells were harvested from 30 mL of cultures by centrifugation at 5,000 rpm for 10 min, resulting in 0.283 g and 0.270 g,

for AG60 and AG10, respectively. The resulting cell pellets were microwave-irradiated two times for 15 s each time. The treated microalgal cell pellets were added to 25 mL of media composed of (per 1 L): 500 mg KH₂PO₄, 400 mg MgSO₄·7H₂O, 400 mg NaCl, 400 mg NH₄Cl, 50 mg CaCl₂·2H₂O, and 2 mg FeSO₄·7H₂O at a final pH of 7.5. A 5 mL aliquot of seed sludge was added to the serum bottles, resulting in the total volume of 30 mL. To assist decomposition of microalgal cells, zero-valent iron (ZVI) powder (12310, Sigma-Aldrich; ranging 45–150 μm) was added to the incubation bottles to a final concentration of approximately 20 g/g of total solid (TS) (microalgae plus seed sludge). Negative controls were prepared with seed sludge and zero-valent iron in the absence of microalgae. The serum bottles were purged with 100% ultrapure N₂ gas for 20 min and sealed with butyl rubber stoppers. Triplicate serum bottles for each treatment (AG60, AG10 and the negative control) were incubated at 37 °C, with shaking at 150 rpm.

Analysis of methane

Head space gas was retrieved from the serum bottles at the selected time points using 21 G syringe needles and transferred to 0.6-L Tedlar gas sampling bags (30284-U, Sigma-Aldrich) or water-filled and rubber-stoppered 15-mL serum bottles. The over-pressurized volumes were recorded by reading the syringes for later calculation of the total CH₄ volume at each time point. Methane gas was analyzed by gas chromatography (GC) using an Agilent Technologies 7890 A GC (Santa Clara, CA, USA) equipped with a flame-ionization detector (FID). Methane calibration gas samples of 10 ppm, 100 ppm, 1,000 ppm, 1%, 10%, and 99% were used. Methane concentrations were converted to the volume produced in the headspace, at the selected sampling time points, by considering the pressure present in each serum bottle using the following equation:

$$\text{Produced methane volume (mL)} \\ = [Hv(\text{mL}) + OPv(\text{mL})] \times GC - CH_4/10^6$$

where *Hv*, *OPv*, and *GC-CH₄* values are headspace volume, over-pressurized volume, and GC-measured volumetric concentration of methane (ppm), respectively.

Quantitative analysis of the methanogen-specific *mcrA* gene and 16 S rRNA genes

The methanogen-specific *mcrA* gene, encoding the alpha subunit of methyl coenzyme M reductase, was used for comparison and relative quantification of methanogens in the AD incubations. Primer pairs *mcrA_F3* (CTT GAA RMT CAC TTC GGT GGW TC) and *mcrA-rev* (CGT TCA TBG CGT AGT TVG GRT AGT) were used for quantitative PCR (qPCR) with the expected product

Table 1 Measurements of total solids (TS) and volatile solids (VS)

Component	TS (g/L)	VS (g/L)	pH
AG-60,784	9.444 ± 0.473	8.578 ± 1.951	7.36
AG-10,047	9.000 ± 0.693	5.578 ± 1.902	7.22
Seed sludge	38.378 ± 2.500	22.956 ± 2.041	7.51

size of 470 bp [16]. As an internal comparison, the 16 S rRNA gene was also analyzed for relative quantification using primer pair of 1369 F (CGG TGA ATA CGT TCY CGG) and 1492R (GGW TAC CTT GTT ACG ACT T) with the expected product size of approximately 140 bp [17]. DNAs were extracted from initial and the final incubation sludges using DNeasy PowerSoil Pro Kits (Qiagen, Venlo, Netherlands). All the qPCR experiments were performed on Bio-Rad CFX Connect System (Hercules, CA, USA) using AccuPower 2X GreenStar qPCR Master Mix (Bioneer, Daejeon, South Korea).

For absolute quantification of the gene copies, the *mcrA* and 16 S rRNA genes were amplified in multiple reactions by conventional PCR using the same primers used for qPCR and pooled into each tube. The DNA concentrations were quantified using Qubit Fluorometer 3 (Invitrogen, Waltham, MA), and serially diluted for standardization. A series of concentrations were determined for C_q values and standard curves were constructed to be used for calculating unknown concentrations of the genes. The gene copy numbers in a series of concentrations were calculated theoretically based on the below equation [18]:

$$\begin{aligned} \text{Gene copies} = & [\text{DNA concentration}(\text{ng}/\mu\text{L}) \\ & \times \text{Avogadro's number}]/[\text{DNA size (bp)} \\ & \times \text{molecular weight of dsDNA (approx. 660)}] \end{aligned}$$

Sequence analysis of microbial community structure

The extracted and quantified DNAs were used for sequence analysis of 16 S rRNA genes using an iSeq 100 system (Illumina, San Diego, CA, USA). The v4 region of 16 S rRNA gene was amplified using the primer pair of 533 F (GTG CCA GCM GCC GCG GTA A) and 806R (GGA CTA CVS GGG TAT CTA AT). Following the purification using AMPure XP (Beckman Coulter, Brea, CA, USA) and indexing with Nextera XT DNA Library Preparation Kit (Illumina), the standardized and pooled DNAs were sequenced using iSeq 100 i1 Reagent v2 (Illumina). The generated fastq data were analyzed using mothur ver. 1.48.1 [19] and using Silva database 138.2 [20] for sequence alignment and RDP database 19 [21] for taxonomy assignment as described [22]. Operational taxonomic units (OTUs) were clustered at a 97% sequence similarity.

Linear discriminant analysis effect size (LEfSe) analysis was performed to identify the specific microbial taxa responsible for the community shifts between the initial sludge and the final AD sludges using mothur, and visualized using the ggplot2 package of R. Permutational multivariate analysis of variance (PERMANOVA) was performed using the adonis2 function in the vegan

package with 999 permutations. Homogeneity of dispersion was assessed using the betadisper function.

Results and discussion

Methane production from anaerobic digestion of microalgae

The AD of seed sludge (average value of 0.11 g VS) and microalgal cells (0.26 g VS and 0.17 g VS, for AG60 and AG10, respectively) showed significant differences in methane gas production between control (done only with the seed sludge; 3.16 ± 0.24 mL at 12 d) and microalgae incubations (12.3 ± 1.6 mL and 15.8 ± 0.7 mL, respectively for AG60 and AG10 at 12 d) (Fig. 1A). The average methane volumes in the controls without algal cells, which were considered as methane produced only from seed sludge, were subtracted from volumes generated at each time point. By converting the values based on VS only from algal cells, the generated methane concentrations were 35.7 ± 6.3 mL/g VS for AG60 and 75.5 ± 4.2 mL/g VS, respectively for AG60 and AG10 at 12 d (Fig. 1B). The methane volumes generated at each time points were summed to be the accumulated methane volumes, which were 93.2 ± 8.7 mL/g VS (seed sludge only), 139.7 ± 8.4 mL/g VS (algal cells only), and 261.7 ± 6.1 mL/g VS (algal cells only), respectively for the control, AG60, and AG10 (Fig. 1C).

In general, the theoretical efficiency of methane production from microalgae AD has been estimated to be approximately 300–500 mL/g VS based on the theoretical yields of methane from lipids, proteins, and carbohydrates [23]. Moreover, the actual monitored efficiency has been reported to range from approximately 100–300 mL/g VS [24, 23]. It varies due to many factors including microalgae species and incubation conditions, such as pre-treatment, mechanical, chemical, and/or thermal factors.

While a direct ZVI-free control for microalgae was not included in the experimental design, a comparative analysis with historical data confirms the enhancement effect of ZVI. Previous studies report that the methane yield of untreated *Scenedesmus* (synonym of *Tetradesmus*) biomass typically ranges between 107 and 186 mL CH₄/g VS due to cell wall recalcitrance [25, 26]. In this study, the AG10 strain supplemented with ZVI achieved a cumulative methane yield of 261.7 mL/g VS, which is statistically superior to the empirical maximums reported for untreated biomass. This comparison serves as a literature-based control framework, suggesting that ZVI addition effectively overcame the intrinsic limitations of algal biomass conversion.

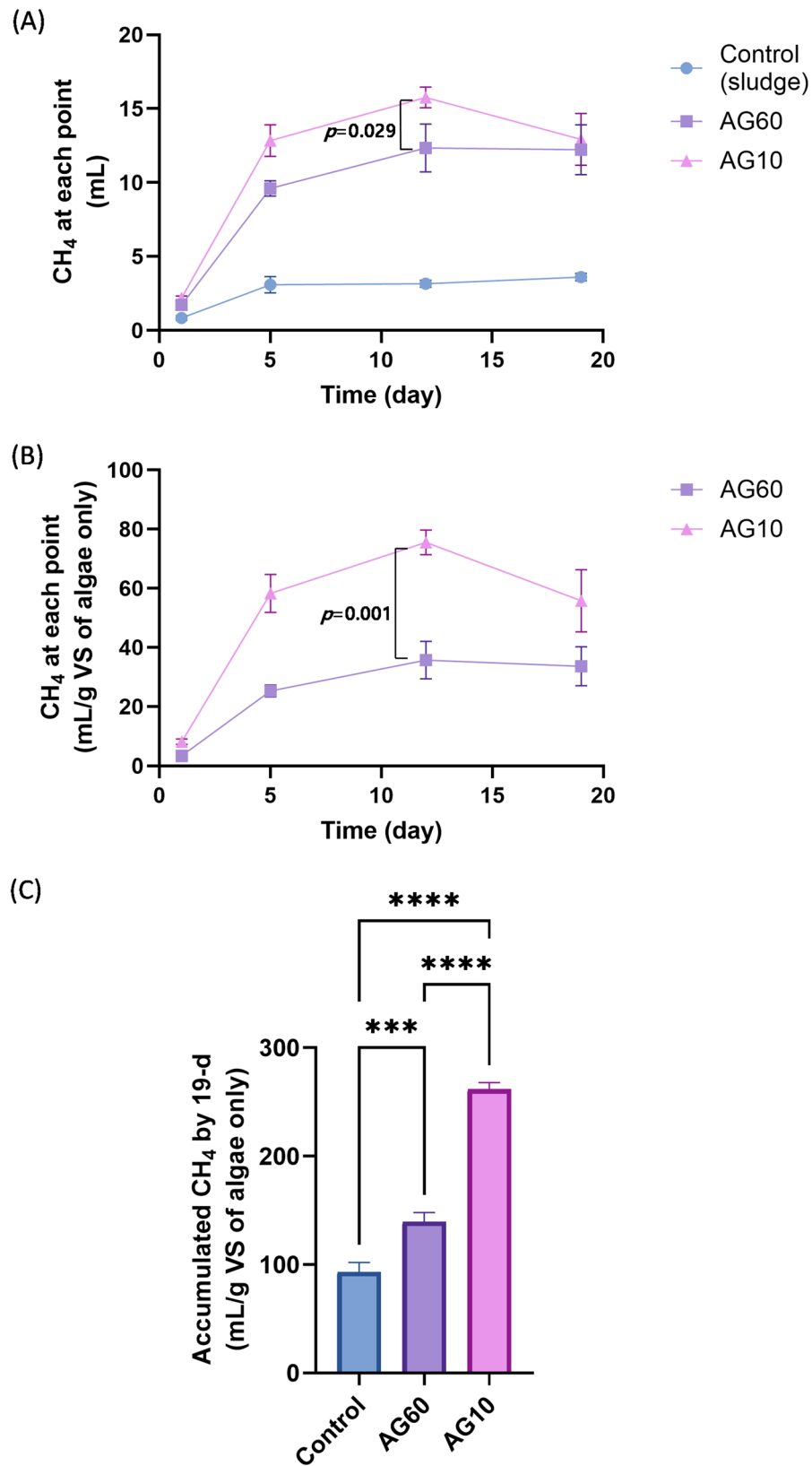


Fig. 1 Methane production at the selected time points over the incubation period. **(A)** methane in 1 atm-headspace; **(B)** methane per VS (algae); **(C)** accumulated methane per VS (algae). *Control is based on the sludge VS value only. Error bars represent standard deviations of triplicate. Statistics indicate *p*-values by t-test **(A, B)**, and ANOVA with Tukey's multiple comparison test **(C)**, *** *p* = 0.0008; **** *p* < 0.0001

Quantitative analysis of methanogen-specific and 16 S rRNA genes

Results in Fig. 2 show qPCR results of the absolute gene copy numbers of 16 S rRNA and *mcrA* genes in the sludge samples. After 19 days of incubation, the average relative abundance of 16 S rRNA genes (calculated from triplicate bottles for each treatment) decreased compared to the initial sludge. Specifically, the final abundances were 0.51, 0.86, and 0.78 times the initial level, respectively for the control, AG60, and AG10 treatments (Fig. 2A). This decrease could be attributed to the effects of batch incubation, which limit nutrient availability. However, the decrease was notably less severe in treatments using microalgae cultures compared to the control, suggesting that microalgal biomass served as nutrient and carbon sources that supported greater microbial biomass. Also, it may be possible that 16 S rRNA genes from microalgal chloroplasts and mitochondria have been incorporated in the results within the microalgae-fed treatments, although it is less likely to be biased by mitochondrial and chloroplast DNAs derived from the microalgae themselves due to the specific primers targeted to bacterial and archaeal sequences.

The relative abundance of *mcrA* gene, used as a marker for methanogen indicated that the final gene copies were 1.07, 1.72, and 1.15 times higher than the initial sludge, respectively for the control, AG60, and AG10 treatments (Fig. 2B). Morris et al. [27] demonstrated a positive correlation between abundance of *mcrA* gene and methane production in anaerobic digestion, and Doloman et al. [28] used *mcrA* gene-specific primer set for NGS analysis, suggesting that a single-species, *Methanosarcina mazei*, dominated the methanogenic system at the last stage of AD. Consistent with these molecular observations, methane production also differed among the treatments in this study. The relatively high *mcrA* gene abundance in AG60 corresponded to its cumulative methane yield, compared to the control. This alignment between methanogen increase and methane output suggests that substrate characteristics such as the degradation of algal biomass in AG60 facilitated favorable conditions for methanogenic activity. In contrast, although AG10 showed less enriched *mcrA* abundance, the magnitude of methane yield was larger, reflecting a multiple factors-influencing AD system. Together, these results indicate that differences in microalgal biomass composition directly influenced methanogen dynamics and, consequently, methane productivity.

The ratio of *mcrA*/16S rRNA genes was calculated to evaluate the relative dominance of methanogens within the total microbial community (Fig. 2C). The initial sludge exhibited a ratio of 0.072. Following the 19-day incubation, this ratio increased across all groups, reaching 0.177 in the control, 0.141 in the AG60 treatment,

and 0.111 in the AG10 treatment. These values represent an enrichment of the methanogenic population relative to the total bacteria and archaea by factors of 2.45, 1.95, and 1.53 times the initial level for the control, AG60, and AG10, respectively. While the control showed the highest numerical ratio, this was primarily due to the sharpest decline in the total 16 S rRNA gene rather than a higher absolute abundance of methanogens. In contrast, the microalgae-supplemented groups maintained higher absolute gene counts for both the genes. It must be noted that while *mcrA* is typically single-copy, 16 S rRNA operons vary from 1 to 15 copies per genome in bacteria. Therefore, the ratio is an index of enrichment, not an exact cell-to-cell ratio.

The shift in the *mcrA*/16S rRNA ratio provides critical insight into the microbial succession within the reactors. The increase from an initial 0.072 to as high as 0.177 (control) and 0.141 (AG60) demonstrates that the anaerobic digestion environment successfully favored the proliferation or survival of methanogenic archaea over the broader bacterial community. In the control group, the high ratio of 0.177 likely reflects a substrate deficient state where the total microbial community (by 16 S rRNA gene) collapsed significantly (to 0.51 times the initial level, Fig. 2A), leaving a small but persistent methanogenic population (1.07 times, Fig. 2B). Conversely, the AG60 group maintained a more balanced and robust community. With a *mcrA*-to-16 S ratio of 0.141 and a significantly higher retention of total microbes (0.86 times the initial level), AG60 represents a more stable and productive system. This suggests that the chemical composition of the AG60 microalgae specifically supported a relatively high-density methanogenic environment. The slightly lower ratio in AG10 (0.111) compared to AG60 indicates that the AG10 biomass was less efficient at fostering methanogen enrichment, though it is not well aligned with its higher cumulative methane yield (Fig. 1). These results underscore that *mcrA*/16S rRNA gene ratios could indicate methanogenic niche establishment.

Microbial community analysis

A total of 3,004,693 sequences were generated from analysis of DNAs from the 12 AD samples of the initial sludge and final sludges from the control, AG60, and AG10 treatments (triplicates). After removing sequencing errors and chimera-containing sequences a total of 1,929,636 sequences were acquired, which clustered into 237,277 OTUs. The OTUs were normalized to the smallest number in the samples (34,302).

Results in Fig. 3 show the relative abundance of bacteria at the phylum and genus levels. There were changes in the proportions of several taxa between the initial and the final samples. The relative abundances of members of the phylum *Actinomycetota* at the final stage were

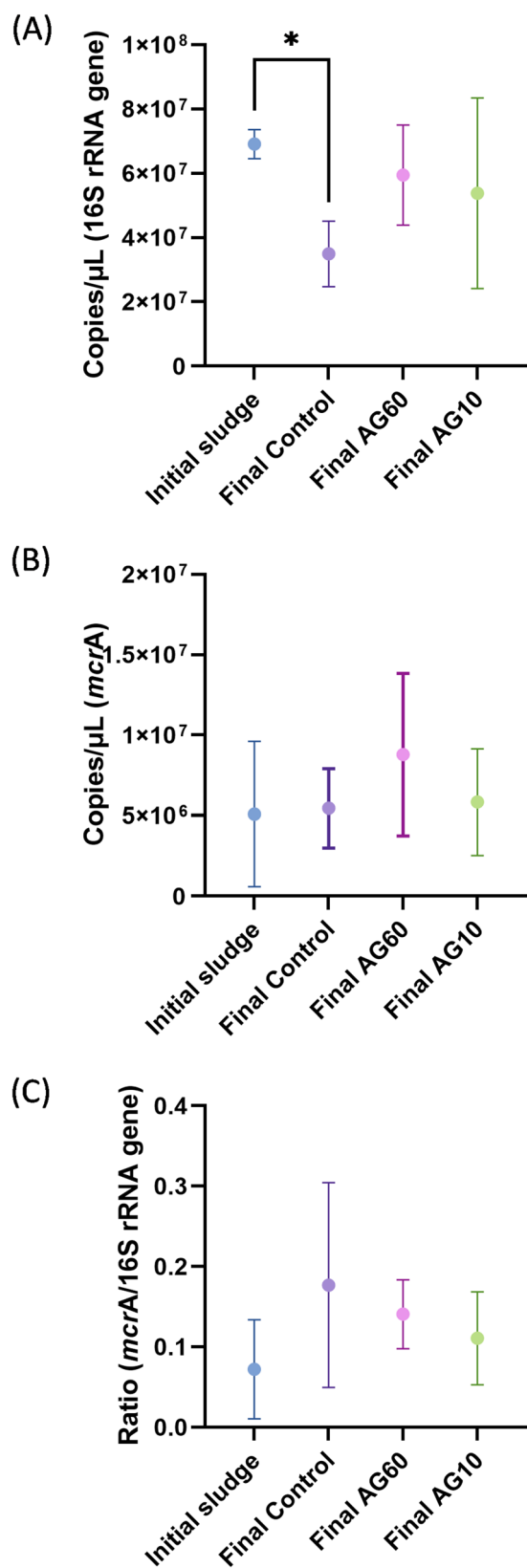


Fig. 2 Absolute copy numbers of 16S rRNA gene (A) and *mcrA* gene (B), and the *mcrA*/16S rRNA gene ratio (C) for the initial seed sludge and the final incubation sludges from the three treatments. Error bars represent the standard deviation of triplicate measurements. An asterisk denotes a statistically significant difference as determined by a t-test ($p < 0.05$)

$20.6 \pm 0.7\%$, $17.7 \pm 5.3\%$, and $20.4 \pm 1.1\%$ for the AG10, AG60, and control samples, respectively, and $9.2 \pm 0.4\%$ for the initial sludge samples. While the percentages of *Bacillota* ($23.3 \pm 1.3\%$, $24.4 \pm 3.3\%$, $15.3 \pm 1.6\%$, and $6.7 \pm 0.5\%$) and *Pseudomonadota* ($19.5 \pm 0.3\%$, $18.4 \pm 2.0\%$, $24.4 \pm 1.3\%$, and $16.8 \pm 0.3\%$) increased from initial levels in the final sludges (Fig. 3A), members of the phylum *Bacteroidota* ($1.6 \pm 0.2\%$, $6.3 \pm 1.8\%$, $2.1 \pm 0.5\%$, and $14.1 \pm 0.3\%$) showed a decrease.

Increases in members of the phylum *Bacillota* were larger in the AD incubations with algal cells, than in controls without algal cells. Members of this phylum are known to contain efficient cellulolytic bacteria including *Clostridium*, *Bacillus*, and *Paenibacillus* [29, 30]. Since microalgal cells provide cellulose compounds, this is likely providing growth substrate for cellulolytic microorganisms.

At the genus level, the relative abundances of *Anaerolineaceae* (unclassified), *Mycobacterium*, *Proteiniborus*, *Romboutsia*, and *Bacillota* (unclassified) increased relative to the initial sludge (T0), while members of the unclassified *Bacteria*, *Bacteroidota*, and *Bacteroidales* showed decreases (Fig. 3B). The relative increases in the abundance of *Anaerolineaceae* (unclassified) were larger in the algal incubations than in AD controls without algal cells. Members of the family *Anaerolineaceae* has been known to play a key role in AD and the anaerobic metabolism of long-chain alkanes [31]. The increases of specific taxa suggest that members of the family *Anaerolineaceae* may have participated in the degradation of complex algal biomass, likely through syntrophic interactions with methanogens.

The complexity of AD processes involves several stages (hydrolysis, acidogenesis, acetogenesis, and methanogenesis) driven by diverse microbial populations acting as a consortium [1]. Each stage involves distinct microbial communities and reactions, and therefore it would be important to understand the microbial communities of each AD stage to enhance methane production, particularly of hydrolysis and methanogenesis stages as they are often rate-limiting. As shown in the bacterial community analysis, the increase in cellulolytic bacteria, particularly those within the phylum *Bacillota*, suggests the efficient degradation of microalgal biomass. Microorganisms

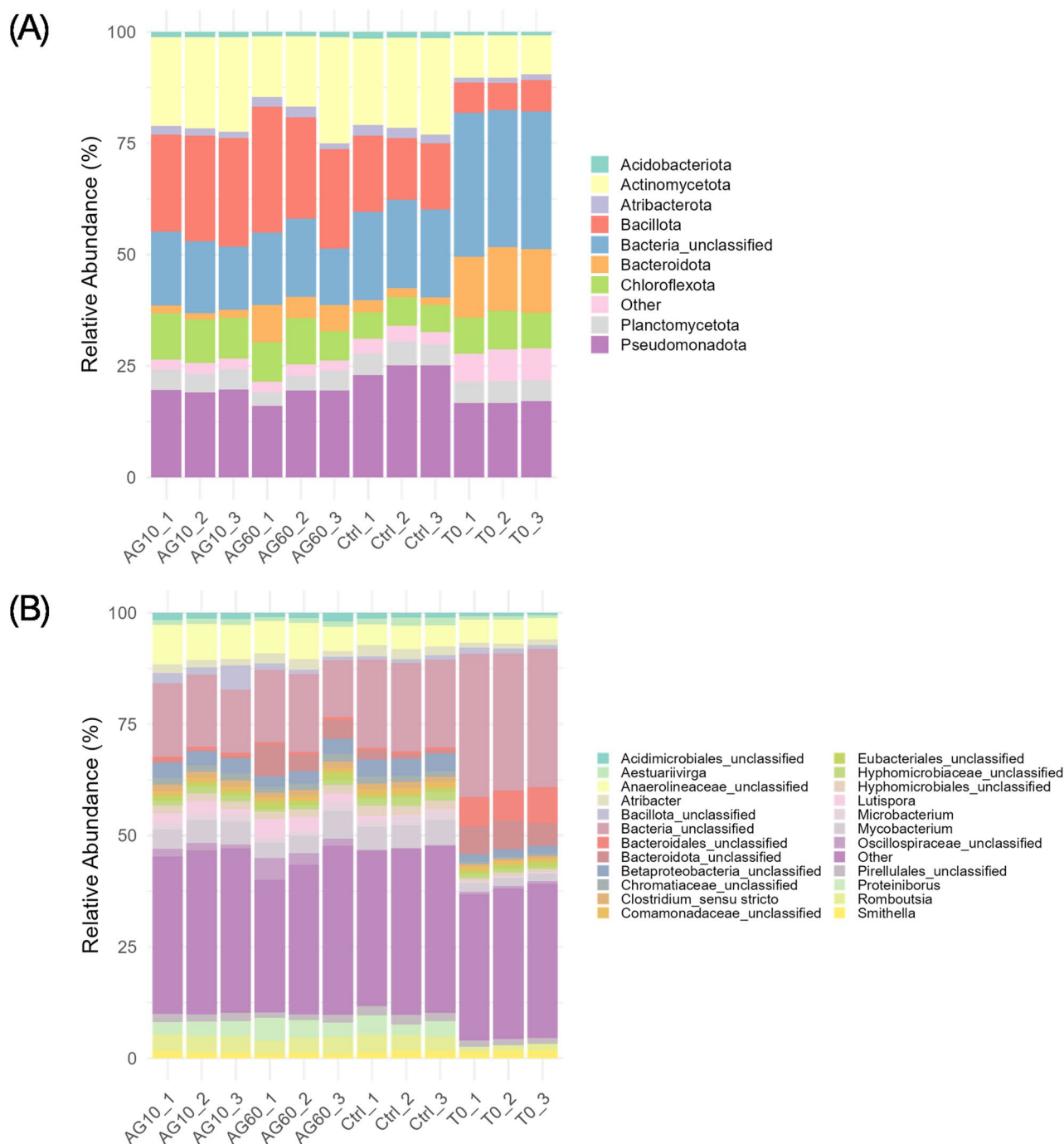


Fig. 3 Relative sequence abundance of *Bacteria* at the (A) phylum and (B) genus levels. Taxa with a total percentage (sum of all samples) of less than 1%, binned into "Other"

capable of degrading cellulose and other complex organic materials into simpler, soluble compounds are very important in the hydrolysis stage, especially with microalgal biomass, and may affect overall AD efficiency.

The relative abundances of *Archaea* at the phylum and genus levels are shown in Fig. 4. Most of *Archaea* were from the phylum *Euryarchaeota* with an average abundance $97.7 \pm 2.3\%$ across the samples (Fig. 4A).

This includes the majority of well-known methanogens, including members of the genera *Methanobacterium*, *Methanococcus*, and *Methanosarcina* [32]. Indeed, there were significant increases in the abundance of members of the genus *Methanobacterium* in the final samples ($43.6 \pm 12.4\%$, $59.9 \pm 7.1\%$, and $46.1 \pm 2.3\%$, respectively for AG10, AG60, and the control), relative to the initial sludge (T0, $9.7 \pm 0.9\%$) (Fig. 4B). Also, the unclassified

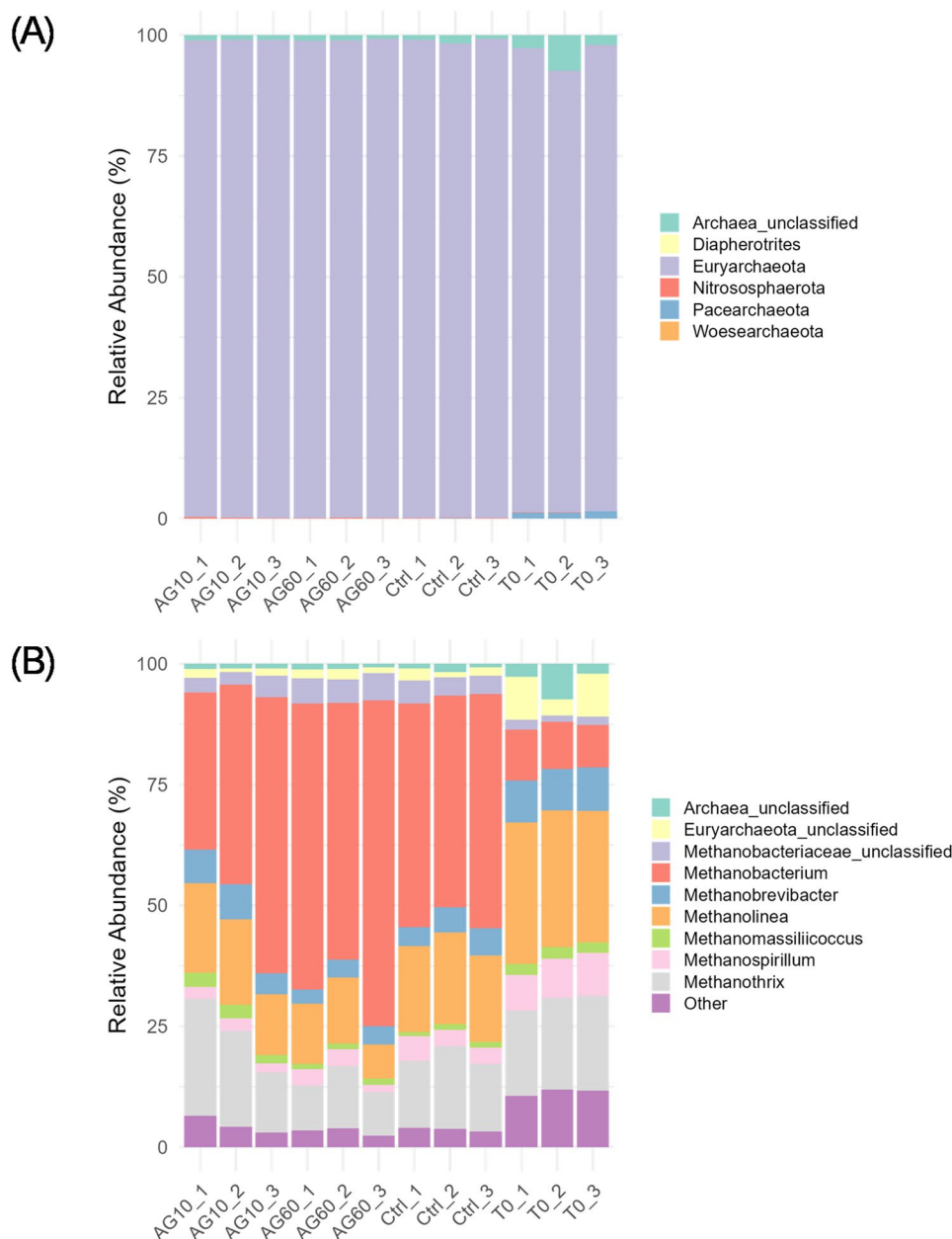


Fig. 4 Relative sequence abundance of *Achaea* at (A) phylum and (B) genus levels. Taxa with a total percentage (sum of all samples) of less than 1%, binned into "Other"

Methanobacteriaceae increased after incubations, regardless of microalgal addition ($3.4 \pm 1.0\%$, $5.2 \pm 0.4\%$, $4.1 \pm 0.5\%$, and $1.7 \pm 0.4\%$, respectively for AG10, AG60, the control, and T0). In contrast, the hydrogenotrophic methanogens, such as *Methanolinea*, *Methanobrevibacter*, and *Methanospirillum*, showed decreases after the incubations (Fig. 4B). In addition, the acetoclastic methanogen *Methanothrix* [33] showed a relatively smaller decrease, or stable abundance in AG10 compared with the initial relative abundance (Fig. 4B).

Methanogenesis in anaerobic digestion is primarily driven by three types of methanogens: hydrogenotrophic,

acetoclastic, and methylophilic types. Each contributes uniquely to methane production, with their relative importance depending on environmental conditions and substrate composition, such as municipal wastewater, food waste, agricultural residues, or manure. Methylophilic methanogens play a crucial role in the final stages of organic matter degradation in anaerobic environments. Unlike other methanogens that use hydrogen or acetate, these specialized archaea utilize methylated compounds such as methanol and methylamines. In comparison, acetoclastic methanogens often dominate environments with elevated acetate concentrations, such

as sewage sludge and certain agricultural wastes [34–36]. In contrast, hydrogenotrophic methanogens tend to prevail when hydrogen is more readily available. Under high organic loading conditions, acetoclastic methanogens can significantly enhance methane yields, making the acetoclastic pathway a key contributor to methane production [37]. In this current study, however, where high organic loading conditions were unlikely, the predominance of hydrogenotrophic methanogenesis can be assured by the dominance of *Methanobacterium*, a genus known to utilize exclusively H₂ and CO₂ to produce methane. Unlike some other methanogens like *Methanosarcina*, which can perform both hydrogenotrophic and acetoclastic methanogenesis, *Methanobacterium* lacks this metabolic flexibility to use acetate or other methylated compounds [32]. Given the presence of diverse cellulose-degrading bacteria (Fig. 3), it is likely that organic matter, including microalgal cells, was extensively degraded to CO₂ by heterotrophic microorganisms, subsequently fueling hydrogenotrophic methanogenesis.

The predominance of *Methanobacterium* in the ZVI-amended groups can be mechanistically explained by the corrosion of ZVI ($\text{Fe}^0 + 2\text{H}_2\text{O} \rightarrow \text{Fe}^{2+} + \text{H}_2 + 2\text{OH}^-$), which provides a direct supply of dissolved hydrogen as a substrate for hydrogenotrophic methanogens. Furthermore, the release of hydroxide ions contributes to pH buffering, mitigating acidification during rapid algal hydrolysis. The strongly reducing environment created by ZVI (lowering ORP) favors methanogenesis, and conductive iron particles may have facilitated direct interspecies electron transfer (DIET) between syntrophic bacteria and methanogens, bypassing diffusive hydrogen transfer and enhancing efficiency. This aligns with the observed enrichment of *Methanobacterium*, a genus known to utilize H₂ and potentially participate in DIET.

Microbial community analysis revealed changes in both bacterial and archaeal populations during AD. We

observed an increase in a single genus of methanogen, *Methanobacterium*, which implies obvious production of methane and dominance of hydrogenotrophic conditions. The result clearly showed that most complex organic materials were completely degraded to CO₂, otherwise some methanogens capable of acetoclastic methanogenesis in circumneutral temperatures, such as *Methanosaeta*, *Methanosarcina*, etc. would have appeared together in the final stages of AD [38].

Analyses of alpha diversity, done using Chao1, Shannon, and inverse Simpson indices, indicated that there were no significant changes of observed species (OTUs) between the initial sludge and the final sludge samples across three treatment groups (Table 2). These results suggest that the overall taxonomic richness was maintained throughout the anaerobic digestion process. However, the Shannon index in group AG60 showed a significant decrease from the initial sludge samples (6.756 ± 0.076 , T0) to 6.480 ± 0.047 ($p = 0.006$), and similar declining trends were observed in the Inverse Simpson index (Table 2). This reduction in diversity indices, particularly in the microalgae-fed reactors, likely reflects the selective enrichment of specific functional groups involved in algal biomass degradation and methanogenesis.

To further identify the specific microbial taxa responsible for the community shifts, LEfSe analysis was performed to compare the initial sludge with the final AD sludges (Fig. 5). The analysis revealed that several key OTUs were significantly enriched in the final stages, acting as biomarkers for the stabilized AD process. Notably, the hydrogenotrophic methanogen *Methanobacterium* (OTU 1) and the acetoclastic methanogen *Methanotherix* (OTU 12) showed high LDA scores, confirming their dominant roles in methane production. Additionally, proteolytic and cellulolytic taxa such as *Proteiniborus* (OTU 5, 25), *Romboutsia* (OTU 4), and *Lutispora* (OTU

Table 2 Alpha-diversity indices of OTUs. Statistical significance was evaluated using one-way ANOVA followed by Bonferroni-adjusted post hoc pairwise comparisons among groups (a, $p = 0.006$; b, $p = 0.026$; c, $p = 0.028$)

Group	Sample (replicate)	Good's coverage	Observed species (OTUs)	Alpha-diversity index		
				Chao1	Shannon	Inverse Simpson
AG10	AG10_1	0.807 ± 0.001	8,110 ± 39	55,852 ± 1,901	6.790 ± 0.008	137.2 ± 1.3
	AG10_2	0.851 ± 0.001	6,598 ± 43	34,258 ± 1,265	6.496 ± 0.010	118.0 ± 1.3
	AG10_3	0.843 ± 0.002	6,862 ± 50	37,921 ± 1,527	6.501 ± 0.012	104.0 ± 1.3
AG60	AG60_1	0.814 ± 0.002	7,727 ± 68	52,226 ± 2,300	6.426 ± 0.016 ^{a, b}	84.0 ± 1.3 ^c
	AG60_2	0.834 ± 0.000	7,173 ± 0	40,503 ± 0	6.503 ± 0.000 ^{a, b}	107.7 ± 0.0 ^c
	AG60_3	0.831 ± 0.002	7,289 ± 54	42,981 ± 1,744	6.511 ± 0.013 ^{a, b}	86.2 ± 1.4 ^c
Control	Ctrl_1	0.802 ± 0.002	8,301 ± 68	54,069 ± 2,195	6.776 ± 0.015 ^b	118.5 ± 2.0 ^c
	Ctrl_2	0.834 ± 0.001	7,279 ± 43	38,427 ± 1,337	6.661 ± 0.009 ^b	122.5 ± 1.3 ^c
	Ctrl_3	0.836 ± 0.001	7,156 ± 27	40,311 ± 1,058	6.603 ± 0.006 ^b	115.7 ± 0.7 ^c
T0	T0_1	0.792 ± 0.001	8,806 ± 45	52,736 ± 1,697	6.843 ± 0.009 ^a	112.3 ± 1.2
	T0_2	0.823 ± 0.002	7,762 ± 57	39,416 ± 1,404	6.700 ± 0.013 ^a	113.2 ± 1.6
	T0_3	0.820 ± 0.001	7,924 ± 25	39,656 ± 854	6.724 ± 0.005 ^a	115.5 ± 0.7

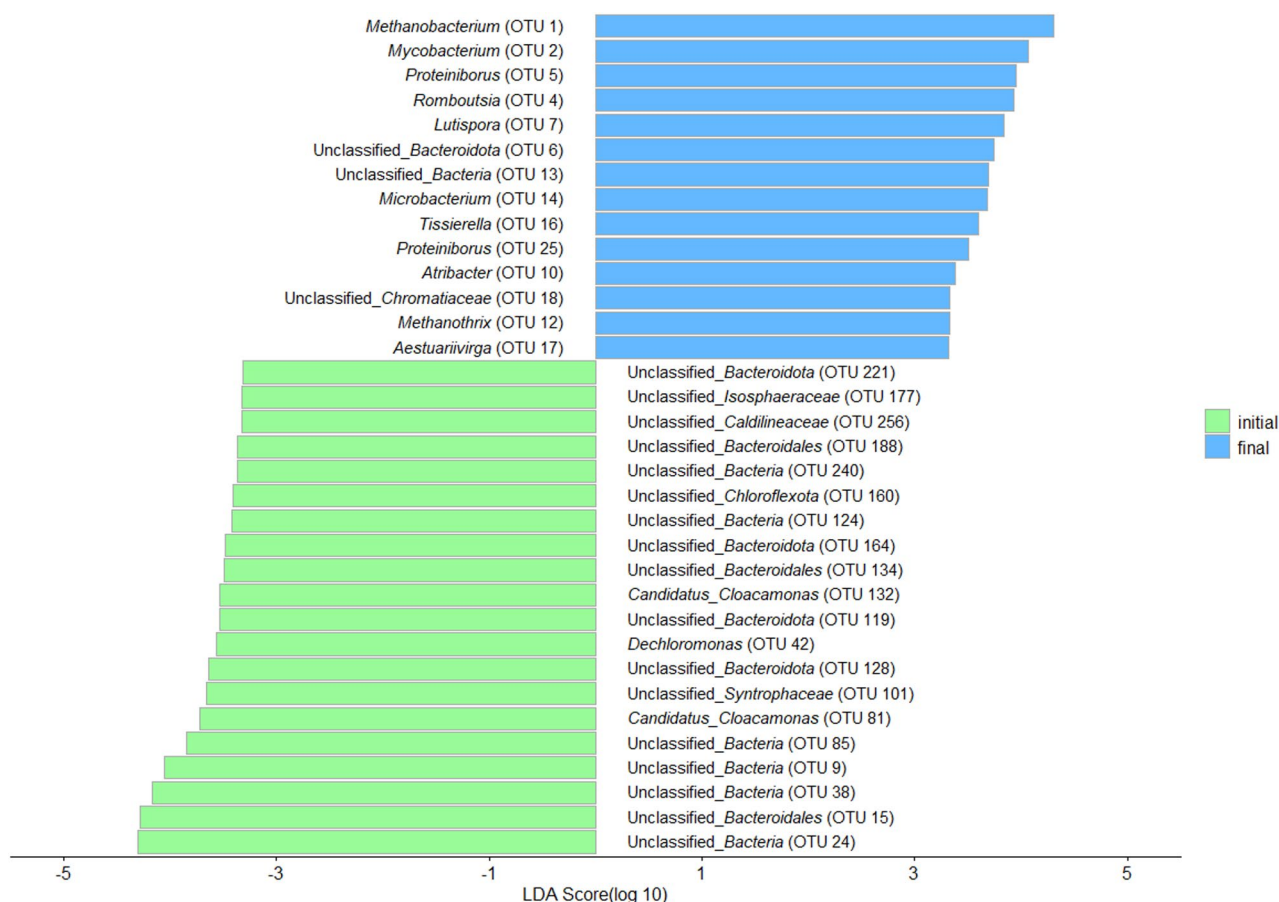


Fig. 5 Differentially abundant bacterial taxa between the initial and final samples identified by LEfSe analysis. Taxa with an LDA score greater than 3.3 (log10) and a statistically significant difference ($p < 0.05$) are displayed. Bars represent the effect size of each taxon, with positive values indicating enrichment in the final group and negative values indicating enrichment in the initial group

7) were significantly enriched, supporting the robust degradation of microalgal biomass. Conversely, members of the *Bacteroidota* (OTU 221, 164) and *Candidatus Cloacamonas* (OTU 132, 81), which were prevalent in the initial seed sludge, significantly decreased by the end of the incubation (Fig. 5).

Sequence-abundance-based heatmaps generated using Bray-Curtis dissimilarity showed clustering of the replicate samples in the bacterial and archaeal analyses (Fig. 6), except for the archaeal phylum-level heatmap, likely due to the lower taxonomic diversity across the samples (Fig. 6C). The bacterial heatmaps (Figs. 6A, B) revealed a clear separation between the microbial communities in the initial and final sludge samples. In Fig. 6A, members of the bacterial phyla *Actinomycetota*, *Pseudomonadota*, and *Bacillota* clustered together. Similarly, genera such as *Anaerolineaceae_unclassified*, *Mycobacterium*, *Proteiniborus*, *Romboutsia*, and *Betaproteobacteria_unclassified* showed close clustering (Fig. 6B). This clustering pattern was also observed among the archaeal taxa, where *Euryarchaeota* was the only dominant phylum (Fig. 6C), and the genera

Methanothrix, *Methanolinea*, and *Methanobacterium* formed a distinct cluster (Fig. 6D). The groupings identified were strongly supported by ordination analyses using three different distance matrices (Fig. 7).

To assess overall shifts in microbial community structure, beta diversity was examined using non-metric multidimensional scaling (NMDS) and principal coordinates analysis (PCoA) based on Bray-Curtis, Jaccard, and Yue-Clayton (thetaYC) distance metrics. Both ordination approaches revealed clear separation between the initial sludge (T0) and the final anaerobic digestion (AD) samples (Fig. 7). Specifically, NMDS ordinations using Bray-Curtis and thetaYC distances showed distinct clustering of T0 relative to the three final treatment groups (Fig. 7A, C), while PCoA results based on all three distance metrics exhibited consistent separation patterns (Fig. 7D, E, F).

Temporal differences in community composition (T0 vs. AG10, AG60, and Control) were further evaluated using PERMANOVA. Significant effects of incubation time were observed across all distance metrics (Bray-Curtis: $R^2 = 0.498$, $F = 9.91$, $p = 0.01$; Jaccard: $R^2 =$

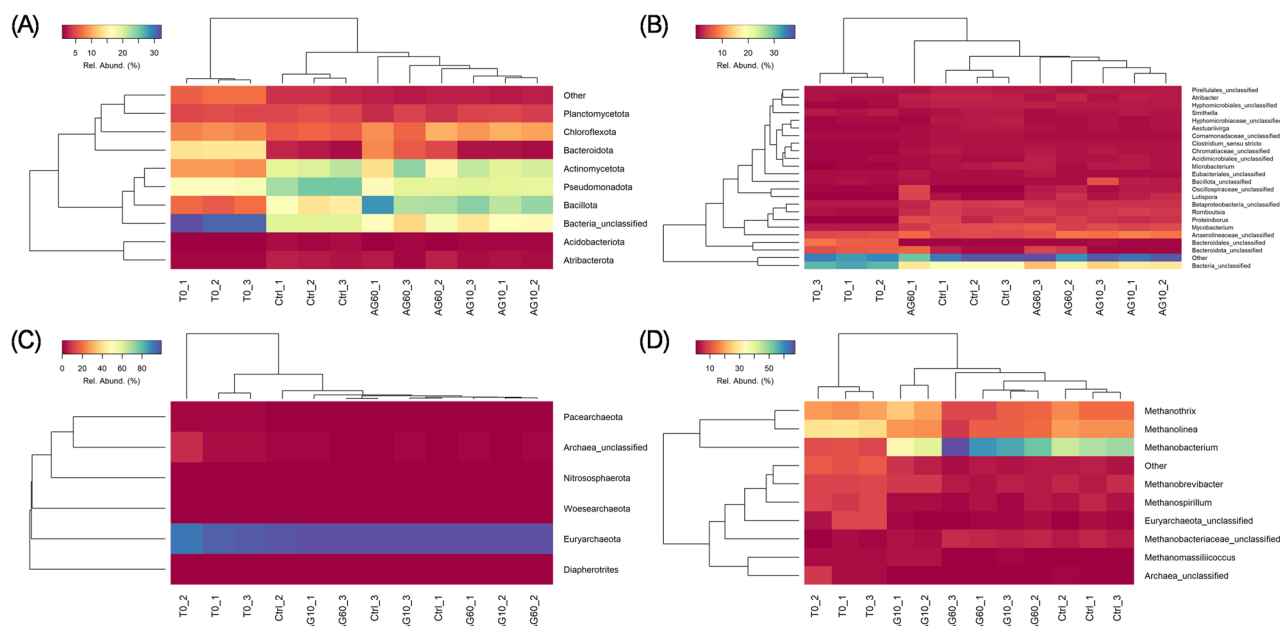


Fig. 6 Relative abundance heatmaps of *Bacteria* (A, B) and *Archaea* (C, D) at the phylum (A, C) and genus (B, D) levels. Dendrograms by Bray-Curtis dissimilarity metrics

0.114, $F=1.28$, $p=0.004$; thetaYC: $R^2 = 0.751$, $F=30.22$, $p=0.005$; Fig. 7). However, tests of multivariate homogeneity of group dispersions indicated significant differences in within-group variance between time points for all metrics (Bray–Curtis: $F=23.47$, $p<0.001$; Jaccard: $F=192.52$, $p<0.0001$; thetaYC: $F=26.63$, $p<0.001$). This suggests that PERMANOVA significance was influenced primarily by changes in community dispersion rather than solely by shifts in group centroids. Accordingly, temporal variation in beta diversity mainly reflects increased community heterogeneity rather than uniform directional compositional changes. Despite these dispersion effects, the consistent clustering of biological replicates across ordination analyses indicates reproducible microbial community succession during the 19-day incubation period.

The spatial separation observed in Fig. 7 is rather consistent with the hierarchical clustering presented in the heatmaps (Fig. 6). In the bacterial analysis, the distinct clustering of final AD sludges away from T0 was primarily driven by the enrichment of specific functional phyla, notably *Bacillota* and *Actinomycetota*, which were identified as key contributors to the stabilized community. At the genus level, the transition in ordination space corresponds to the proliferation of cellulolytic and syntrophic taxa, such as *Anaerolineaceae*_unclassified and *Proteiniiborus*, which facilitated the degradation of the microalgal biomass. Furthermore, the archaeal community showed a dramatic convergence toward a hydrogenotrophic-dominated state, as evidenced by the high relative abundance of *Methanobacterium* in the final stages compared to the

more diverse initial methanogenic population. This shift explains the tight clustering of the final AG10 and AG60 samples in Fig. 7, reflecting the establishment of a specialized methanogenic niche optimized for ZVI-assisted digestion. This result confirms that the addition of microalgal biomass and ZVI imposed strong selective pressure, leading to the formation of a functionally robust and reproducible microbial community optimized for methane production.

Conclusion and perspectives

Since it was previously suggested that the addition of ZVI to the AD process improves the hydrolysis rate of sludge leading to enhanced methane production [39], here we determined whether added ZVI enhanced methane production via decomposition of two selected strains of the green microalga *Tetradismus obliquus*. Results of this study showed that addition of ZVI at 20 g/g TS (total solids of sludge plus algal cells) assisted to produce the accumulated methane of 139.7 ± 8.4 mL/g VS (algal cells only) and 261.7 ± 6.1 mL/g VS (algal cells only), respectively for AG60 and AG10.

Methane production amounts from incubations done using algae AG10 were comparable to or higher than previously reported values [23–26, 39]. This suggests that mildly pre-treated microalgal cells can produce comparable amounts of methane in the presence of zero-valent iron (ZVI) granules. However, the higher methane yield observed for AD of AG10 relative to AG60 suggests differences in biodegradability, most likely due to variations in cell wall composition. Previous studies have also shown

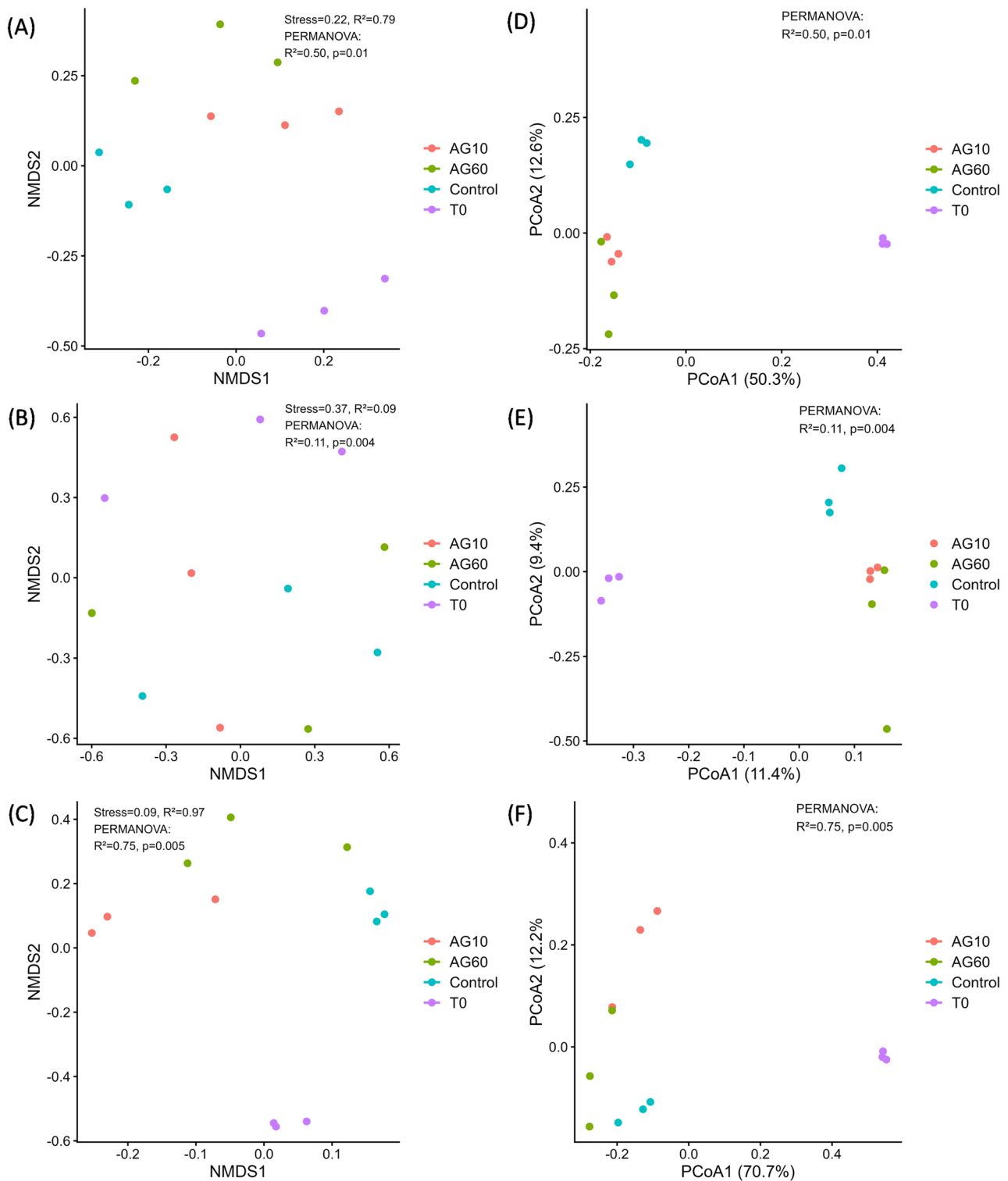


Fig. 7 Ordination analyses of NMDS (A, B, C) and PCoA (D, E, F) based on Bray-Curtis (A, D), Jaccard (B, E), and Yue-Clayton's thetaYC (C, F) distance matrices. PERMANOVA results (R², p-value) indicated within each panel

that microalgae cultivated under nitrogen-limited conditions, which typically lowers protein content, achieved higher methane productivity (462 mL CH₄/g VS/day) than protein-rich microalgae grown in nitrogen-replete media (131 mL CH₄/g VS/day) [12]. These findings indicate that the intrinsic characteristics of microalgal biomass, such as cell wall structure and protein content, play a critical role in anaerobic digestion performance. The results of this study further suggest that careful selection of microalgal species may overcome challenges associated with cell wall recalcitrance and high protein content, thereby enhancing methane productivity even without pretreatment.

To better understand these differences between algal types, future studies may need to include biochemical profiling of algal cells, specifically analyzing lipids, proteins, and carbohydrates, to identify the macromolecules most responsible for enhanced methane production in the presence of ZVI. The underlying mechanism(s) by which ZVI is believed to contribute to increased methane yields remain unclear. Investigating microbial community dynamics and redox conditions, with and without ZVI supplementation, may offer valuable mechanistic insights. Notably, the findings in this study indicate that substantial methane production is achievable with only mild pretreatment, underscoring the need for further research to optimize pretreatment strategies across algal strains to balance energy input and methane yield.

The findings from this study can be further applied to develop an effective monitoring system for AD using molecular techniques such as fluorescence in-situ hybridization (FISH) and quantitative PCR. Moreover, the observed shifts in microbial community composition provide a framework for designing tailored microbial consortia that can enhance biogas production. Moreover, the breakdown of microalgae for methane production might suggest a potential advantage for climate change mitigation, by sequestration of atmospheric CO₂ into microalgal biomass and conversion to renewable bioenergy such as biodiesel, methane, and some valuable organic materials. For such industrial applications, the feasibility of scaling up this process needs to be studied with many variable conditions. Key factors needed to optimize AD processes using microalgal biomass for biogas production include incubation temperature, the use compounds leading to enhanced biomass-degrading, such as ZVI, and presence of stage-specific microbial communities, particularly cellulolytic bacteria and hydrogenotrophic methanogens. Introducing tailored microbial consortia for each phase of the AD process may enhance process efficiency and biogas yields. In addition, since biochemical composition would be different using different groups of green microalgae in the AD process, it might be valuable to evaluate and compare contents

of proteins, lipids, and carbohydrates of different green algae such as *Chlorella*, for carbon fixation efficiency, and biogas conversion efficiency through AD.

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Authors' contributions

Conceptualization, W.-S.K., M.J.S. and J.-H.L.; investigation, S.-Y.Y., W.-S.K., S.-H.K., J.C., J.-H.K., and J.-H.L.; writing—original draft preparation, S.-Y.Y. and J.-H.L.; writing—review and editing, M.J.S. and J.-H.L.; supervision, W.-S.K., M.J.S. and J.-H.L.; project administration, W.-S.K.; funding acquisition, W.-S.K. and J.-H.L. All authors have read and agreed to the published version of the manuscript.

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Data availability

The raw sequences were deposited in the SRA (Sequence Read Archive) of NCBI under BioProject accession number, PRJNA1180725. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests

The authors declare that they have no competing interests. Ji-Hoon Lee is an Editorial Board member of Applied Biological Chemistry.

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