Bioresource Technology 107 (2012) 205–212

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Methane production on thickened, pre-fermented manure

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ARTICLE INFO

Article history:
Received 3 October 2011
Received in revised form 14 December 2011
Accepted 15 December 2011
Available online 29 December 2011

Keywords:
Anaerobic digestion (AD)
Dairy manure
Hydrogenotrophic methanogens
Acetoclastic methanogens
qPCR

ABSTRACT

Over 9 million dairy cows generate an estimated 226 billion kg of wet manure annually in the US. The purpose of this study was to demonstrate the viability of a novel two-stage anaerobic digestion (AD) process for producing methane-rich biogas on pre-fermented dairy manure. In summary, it was observed that AD of thickened pre-fermented manure can generate comparable biogas quantities to AD using raw manure, with enhanced methane content. Despite receiving a lower quality (i.e., partially biodegraded) substrate, biogas stoichiometry and overall process stability in the two-stage system was also comparable to AD receiving raw manure. Finally, the two-stage AD was more enriched with the acetoclastic methanogen *Methanosarcinaceae* (Msc; compared to AD of raw manure) and biogas production appeared closely linked with the Msc fraction. In fact, the enhanced enrichment of Msc likely contributed to the successful and stable operations.

1. Introduction

Over 9 million dairy cows generate an estimated 226 billion kg (249 million tons) of wet manure and produce approximately 5.8 billion kg of CO_2 equivalents annually in the United States (BSSC, 2008; Liebrand and Ling, 2009). Historically lagoons and/or pits have been the most common form of manure management at dairies (Key and Sneeringer, 2011) principally due to ease of construction and operation. More recently, recognizing the potential value of this bio-renewable substrate and also the need to reduce dairy greenhouse gas (GHG) emissions, anaerobic digestion (AD) has been advocated with the goal of producing electricity from methane-rich biogas (Liebrand and Ling, 2009; US EPA, 2010). In fact, the US dairy industry – through the Innovation Center for US Dairy – has committed to reducing GHG emissions 25% by the year 2020 through aggressive construction of ADs (BSSC, 2008), with a stated goal to construct 1300 new AD facilities. However, while AD is being aggressively promoted, the reality is that this technology alone as a commodity production strategy is not anticipated to develop significant economic traction for over 10 years (Zaks et al., 2011). Weak AD economics have in fact proven to be a significant barrier to broad scale deployment. Beyond implementation realities and perhaps most importantly as we seek to maximize value from renewable bio-resources such as manure, AD alone also does not recover all the high value organic matter present in dairy manure (El-Mashad et al., 2008). The work presented herein focuses on establishing a novel AD approach that would integrate within a broader and systematic set of commodity-producing processes to maximize resource recovery and economic value from dairy manure (i.e., including, but not limited to, CH_4 for electricity).

In considering an alternate AD approach, it is necessary to first establish the limitations associated with current AD practices. Conventionally AD is used to process organic matter in a single or two stage configuration. In a single stage system, all three synergistic AD microbial metabolisms – hydrolysis, fermentation, and methanogenesis – occur concurrently within a single tank. However, methane production inefficiencies arise associated with maintaining environmental and growth conditions that intrinsically compromise individual metabolism efficiencies to ensure overall process stability (Khanal, 2008). The inefficient synchronization of the AD metabolisms within the single-stage tank also leaves high-value organic matter unrecovered. Two-stage AD was developed, in part, to remedy single stage metabolism inefficiencies (Ghosh, 1987), in that hydrolysis and fermentation occur in one tank while methanogenesis occurs in a second, downstream tank. In this process configuration VFA-rich supernatant is transferred from the fermentation stage to the second stage methanogenic reactor while residual manure is wasted from the system. The two-stage configuration allows for semi-optimization of the hydrolysis/fermentation and methanogenesis metabolisms, thereby potentially enhancing methane production. However, while two-stage AD can enhance process stability, ultimately this configuration again leaves significant amounts of high value organic matter undigested associated with necessarily shorter retention

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doi:10.1016/j.biortech.2011.12.077

times (RTs) in the hydrolysis/fermentation tanks (El-Mashad et al., 2008).

The research presented and discussed herein was centered on understanding an alternate two-stage AD configuration that, in direct contrast with conventional two-stage AD, digests pre-fermented manure. In other words, the AD process does not receive the VFA-rich supernatant as occurs with traditional two-stage AD. Rather, in this novel configuration the VFA-rich supernatant fraction generated in the hydrolysis/fermentation stage would be recovered to produce other commodities (e.g. bioplastics (Coats et al., 2007), biofuel (Huber et al., 2006)), while the residual, thickened pre-fermented manure would be digested to produce methane-rich biogas for electricity production. In this manner, maximum resource recovery can be realized. To the best of our knowledge this concept of anaerobically digesting thickened pre-fermented organic matter (that is largely depleted of readily hydrolysable carbohydrates and is also without most of the VFA-rich supernatant) has not been investigated or documented. In fact, a principle concern with this AD process configuration would be the ability to even generate measurable quantities of methane on manure that has been pre-fermented. Thus, the specific purpose of the research was to establish process viability and proof of concept for this novel two-stage AD configuration. Research objectives were to: (i) establish bio-methane production potential as contrasted with a conventional single-stage configuration, (ii) establish overall process performance and capability; and (iii) develop an enhanced characterization of the methanogenic populations.

2. Methods

2.1. Source and characteristics of dairy manure

Raw dairy manure was obtained from the University of Idaho dairy farm, located adjacent to the university campus. The university operates an approximate 100–120 head dairy. Ten gallons of fresh manure was collected on a semi-weekly basis and stored at 4 °C until use.

2.2. Experimental design

Two pilot-scale AD systems were designed and operated for the purpose of conducting this research. AD1 was designed and operated as a conventional single stage system (i.e., the control AD), while AD2 represented our novel two-stage system (i.e., coupled fermenter and anaerobic digester). The ADs were constructed of high-density polypropylene, cone-bottomed tanks (Chem-Tainer, West Babylon, NY, USA). The ADs were operated at a design volume of 40 L, and water levels were checked daily to maintain the design volume. Each AD was completely mixed with an Oriental Motors BHF62AT-50 40-watt AC speed control motor (Torrance, CA, USA) equipped with a four-blade 32° pitch-blade turbine operating at approximately 34 rpm. The mixer shaft was inserted into the tank through a 1.9 cm diameter tube set approximately 7.6 cm below the water surface. Each tank was covered using a 1.27 cm thick polypropylene lid to maintain anaerobic conditions inside the digester and to collect biogas. The lids were sealed using a silicone engine sealant and a gasket, and air tested to ensure no leakage. For heating, each AD was fitted with three 15.24 m sections of 0.95 cm diameter copper tubing wrapped around the exterior of the tank, enclosed with insulation. Hot water from a 15.1 L point-of-use water heater was automatically cycled into the tubing every 80 min to maintain mesophilic conditions in the AD. Biogas was vented from the top of each AD through tubing connected directly to wet tip gas flow meters (www.wettipgasmeter.com). Biogas production was recorded in 100 mL increments, and the flow meters were regularly calibrated. Further, the AD biogas vents were regularly switched between the two gas flow meters to ensure that the flow meters did not bias the results. The AD2 system included a 20 L fermenter, which consisted of a polypropylene tank with a flat bottom. The fermenter was completely mixed using a 32° pitched-blade turbine mounted to a 15 W AC motor (USM315-401 W/3GN36KA; Oriental Motors, San Jose, CA).

Both ADs were operated within a temperature range of 35–39 °C; the daily temperature variation was less than 0.5 °C. AD temperatures were recorded continuously using Oakton 4-wire RTD probes (Oakton Instruments, Vernon Hills, Il USA) coupled to a programmable temperature transmitter (Action Instruments, Invensys Eurotherm, Ashburn, VA, USA). AD1 received raw manure, and was operated at a 20 days RT and a design organic loading rate (OLR) of 3.6 g volatile solids (VS; L day)$^{-1}$ (calculated as quantity of VS loaded per day divided by total AD volume (L)). Similarly, the AD2 system (i.e., fermenter and AD) was operated at a 20 days RT (fermenter at 4 days RT, AD2 at 16 days) and a design OLR of 3.6 g VS (L day)$^{-1}$. Raw manure was fed to the fermenter, with thickened fermented residual biomass transferred to AD2. The design OLRs and operating solids content (%, w/w) were within the typical range for ADs receiving organic-rich waste (Khanal, 2008). Since the total volume of the AD2 system was larger, inevitably more manure was processed daily through the combined system (Table 1). Extrapolating to a full-scale scenario, this outcome would be desired in order to maximize use of infrastructure.

The VS content of the manure was monitored regularly, and the quantity of manure fed to each system was modulated as necessary to maintain the design OLR. AD1 was cycled daily by wasting 2 L from the bottom of the digester and adding 2 L of dairy manure homogenized with tap water to maintain the OLR and RT. AD2 was cycled similarly, but 2.5 L was decanted and replaced with residual manure from the fermenter. The fermenter was operated at room temperature (20–21 °C); fermented manure was decanted from the bottom and fed once per day to maintain the target RT. The fermenter waste was centrifuged to recover the residual solids; sufficient supernatant was added to the residual solids to ensure 2.5 L was transferred to AD2. The residual supernatant was used for other research purposes.

2.3. Analytical techniques

Samples were collected to measure the following parameters: total solids (TS), VS, soluble VFAs, pH, and total organic carbon (TOC). For soluble constituents, samples were centrifuged at 10,000 rpm and the supernatant filtered through a 0.22 μm syringe filter (Millipore Corp., Billerica, MA, USA) prior to testing. TS and VS were measured in accordance with Standard Methods 2540G (Clesceri et al., 1998). VFA concentrations were measured using a Hewlett–Packard (Palo Alto, CA, USA) 6890 Series Gas Chromatograph with a flame-ionization detector (FID). The temperature of the column (Grace Davison Discovery Sciences, Deerfield, IL, USA, Alltech® Heliflex® AT™-Wax Column, length 30 m, internal diameter 0.32 mm) was held constant at 150 °C; the injector was maintained at 210 °C and the detector was operated at 210 °C. Helium was used as the carrier gas (flow rate of 1.2 mL min^{-1}). Samples were acidified to a pH of 2 with HCl prior to injection. 0.5 μL of sample was injected in 20:1 split mode for analysis. VFAs were confirmed by matching retention time with known standards (Grace Davison Discovery Sciences, Deerfield, IL, USA) and quantified using linear standard curves ($R^2 > 0.99$). Conversion of VFAs to a COD basis was made using VFA-to-COD stoichiometric ratios (Güngör et al., 2009). pH was measured using a Thermo Scientific Corp. (Waltham, MA, USA) Accumet AP85 waterproof pH/conductivity meter. Dried biomass samples were characterized for TOC

Table 1
Characteristics of the qPCR analyses, including 16S rDNA copy number for the respective methanogens, qPCR annealing temperature, and resultant range of amplification efficiencies for the respective sampling events. Abbreviations as follows: *Methanococcales* (MCC), *Methanobacteriales* (MBT), *Methanomicrobiales* (MMB), *Methanosarcinaceae* (Msc), *Methanosaetaceae* (Mst).

Target group	16S rDNA copy number	qPCR Annealing temperature (°C)	Amplification efficiency (%)
Methanococcales (MCC)	2.86	59.0	72.4–82.9
Methanobacteriales (MBT)	2.5	59.0	87.9–96.4
Methanomicrobiales (MMB)	2.25	59.0	80.2–91.1
Methanosarcinaceae (Msc)	3	56.0	80.5–93.3
Methanosaetaceae (Mst)	2	59.0	66.6–80.9
Archaea	1.8	56.0	83.0–94.7
Prokaryotes	4.1	55.0	76.7–85.3

(Nelson and Sommers, 1982) by the University of Idaho Analytical Sciences Laboratory.

Biogas was characterized using a Gow-Mac (Bethlehem, PA, USA) Series 550P Gas Chromatograph equipped with a thermal conductivity detector (TCD). The temperature of the column (Grace Davison Discovery Sciences, Deerfield, IL, USA, Alltech® Hayesep® DB 100/120 column, 30 ft × 1/8 in. × 0.085 in., stainless steel) was held constant at 100 °C, while the injector was maintained at 174 °C and the detector was operated at 205 °C. Helium was used as the carrier gas (approximate flow rate of 15 mL min^{-1}). One microliter of sample was injected for analysis, collected from the ADs using a gas-tight syringe (SGE Analytical Science, Austin, TX, USA). Gas concentrations were confirmed by matching retention times with known standards (Grace Davison Discovery Science, Deerfield, IL, USA).

2.4. Microbial population analyses

Genomic DNA was extracted from biomass obtained from each AD using the MO BIO PowerSoil® DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA USA). Biomass samples were collected on five dates over the 85 days AD operational analysis period. Quantitative real-time PCR (qPCR) was applied using 16S rDNA-based oligonucleotide primers to estimate the relative abundance of the respective archaeal populations present in the ADs. Specifically, oligonucleotide primers were used to quantify the three principal orders of hydrogenotrophic methanogens (*Methanococcales*, *Methanobacteriales*, *Methanomicrobiales*), and the two most predominant families within *Methanosarcinales* (*Methanosarcinaceae*, and *Methanosaetaceae*). In addition, the relative total archaeal population was quantified as a fraction of the total prokaryotic population. Oligonucleotide forward and reverse primers were designed in accordance with Yu et al. (2005). qPCR was conducted on a StepOne Plus™ Real-Time PCR system (Applied Biosystems, Foster City, CA) using iTaq™ SYBR® Green Supermix w/ROX (Bio-Rad Laboratories Inc., Hercules, CA, USA) with a total reaction volume of 25 μL. qPCR conditions were as follows: 3 min at 95 °C, 45 cycles of 30 s at 95 °C, 45 s annealing (temperatures varied with the primer set; see Table 1), and 30 s at 72 °C. All unknown samples were assessed in triplicate with 5 ng of total genomic DNA and 500 nM final concentration of each primer per reaction (primer concentration was determined through optimization). Selected annealing temperatures (Table 2) were determined through a temperature optimization process. All qPCR melting curves were evaluated to confirm a single melting peak, and agarose gel analysis revealed a single signal for each qPCR primer set. Amplification efficiency was estimated for each primer set using baseline-corrected fluorescence data (from StepOne Software v2.0) with LinRegPCR (Ramakers et al., 2003). The cycle threshold was set at a constant value across all samples based on location within the log-linear region for determination of Cq values (cycle number at which the measured fluorescence exceeds the cycle threshold). Observed amplification efficiencies (Table 1) were comparable to that observed by Yu et al. (2006). Relative microbial abundance (Table 1) was estimated using the mean amplification efficiencies for each primer set, the Cq values for the individual samples, and the 16S rDNA copy numbers. The relative quantity of the respective families/orders was determined according to the ΔΔCq method as described by Pfaffl (2001). For quantification, the 16S rDNA gene copy number for archaea was set at 1.8 (Einen et al., 2008; Lee et al., 2008), while the gene copy number for bacteria was set at 4.1 (He et al., 2007). Copy numbers for each specific methanogenic order/family are listed in Table 1. 16S rDNA gene copy numbers for the different archaeal orders/families were determined using the Ribosomal RNA Operon Database (rrnDB, http://www.rrndb.mmg.msu.edu/).

2.5. Statistical methods

Paired student *t*-tests were used for the statistical comparisons, and differences were declared significant at $p < 0.05$.

3. Results and discussion

3.1. Biogas production and yield

A primary objective of this research was to establish process potential for the proposed novel two-stage AD configuration, most specifically in regards to the ability to generate viable quantities of methane-rich biogas on pre-fermented manure. As context, "viable quantities" would be as compared with conventional AD. Thus, as a control, a conventional single-stage AD (AD1, fed raw manure) was operated concurrently. AD1 and AD2 (fed pre-fermented substrate) were operated continuously for over a year, with performance monitored regularly. The ADs reached steady state early in this operational period, as determined by biogas production. Within this operational period biogas production was recorded continuously for 85 days (duration greater than four RTs), while a more comprehensive analysis of AD performance was made for a 40-day period (equivalent to two RTs) within the 85 days. pH over this operational period remained between 7.4 and 7.6.

While it would appear that biogas production on raw manure slightly exceeded that observed in the two-stage system (Fig. 1 and Table 2), there was no statistical difference in average biogas production over this period between the two ADs ($p = 0.06$). Further, AD2 exhibited less variance in biogas generation (Table 2). Considering that AD-based power producers must strictly adhere to power purchase agreements that typically prescribe narrow operational ranges in electrical production, the observed lower variability in AD2 biogas production would potentially enhance commercial operations. Regarding methane content, the biogas composition for AD1 and AD2 was estimated at 51.4%CH_4: 48.6%CO_2 and 54.3%CH_4:45.7%CO_2, respectively, which are considered relatively typical for AD (Ward et al., 2008). The AD2 methane biogas fraction was determined to be statistically higher than in the AD1 biogas ($p = 1.83 \times 10^{-3}$; Table 2). Therefore, in comparing

Table 2
Summary of operational and performance characteristics and statistics for both anaerobic digesters (AD1 and AD2) over the full operational period.

		AD1	AD2
Average reactor operating TS	% (w/w)	4.8	4.6 (AD2); 4.5 (fermenter)
Average influent fermenter VS	g VS/day	–	208 (n = 25)
Average influent AD VS	g VS/day	148 ± 16 (n = 25)	168 ± 18.3 (n = 25)
Average VS destruction	%	43.7 ± 8.1 (n = 25)	40.6 ± 6.7 (n = 24; AD only) 51.6 ± 7.4 (n = 24; fermenter + AD)
Average biogas produced	L/day	54.5 ± 9.1 (n = 76)	51.8 ± 7.9 (n = 76)
Average methane content	%	51.4 ± 1.1 (n = 28)	54.3 ± 1.2 (n = 28)
Yield	L biogas/g VS destroyed	0.84	0.76
	L biogas/g VS applied	0.37	0.31
	L biogas/L d	1.36	1.30
	L CH_4/L d	0.70	0.71
Influent VFAs	mg/L as COD	2625 ± 1042 (n = 63)	5667 ± 2576 (n = 68)
	mg/L as VFAs	1876 ± 731 (n = 63)	3283 ± 1379 (n = 68)
Effluent VFAs	mg/L as COD	907 ± 564 (n = 63)	1116 ± 809 (n = 69)
	mg/L as VFAs	697 ± 448 (n = 63)	846 ± 670 (n = 69)

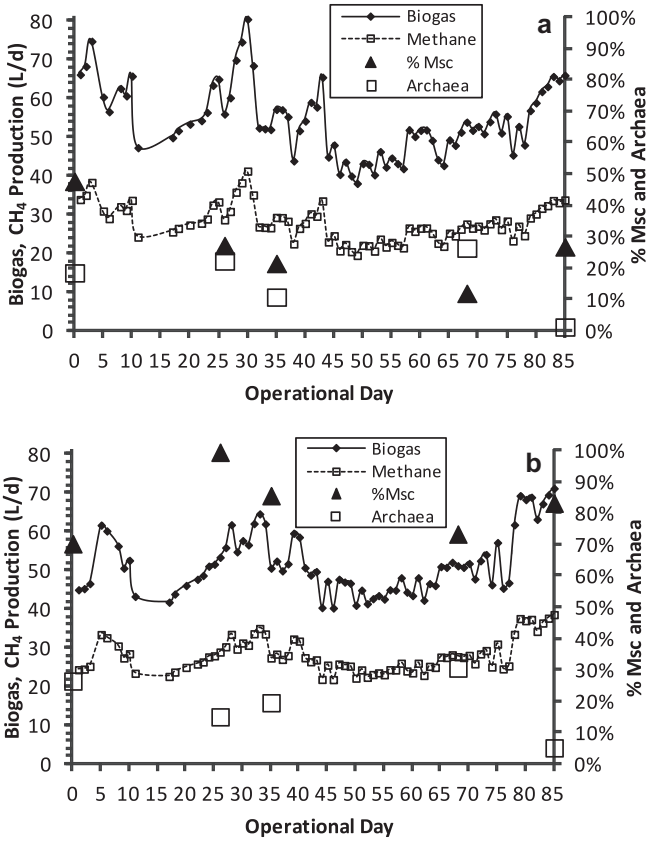

Fig. 1. Daily biogas and methane production over the operational analysis period for: (a) AD1, and (b) AD2. Also shown is the relative fraction of *Methanosarcinaceae* (Msc; as a fraction of the total archaeal population) and the relative fraction of archaea (as a fraction of prokaryotes) present in the respective AD biomass. Pertinent statistics are presented in Tables 1 and 3.

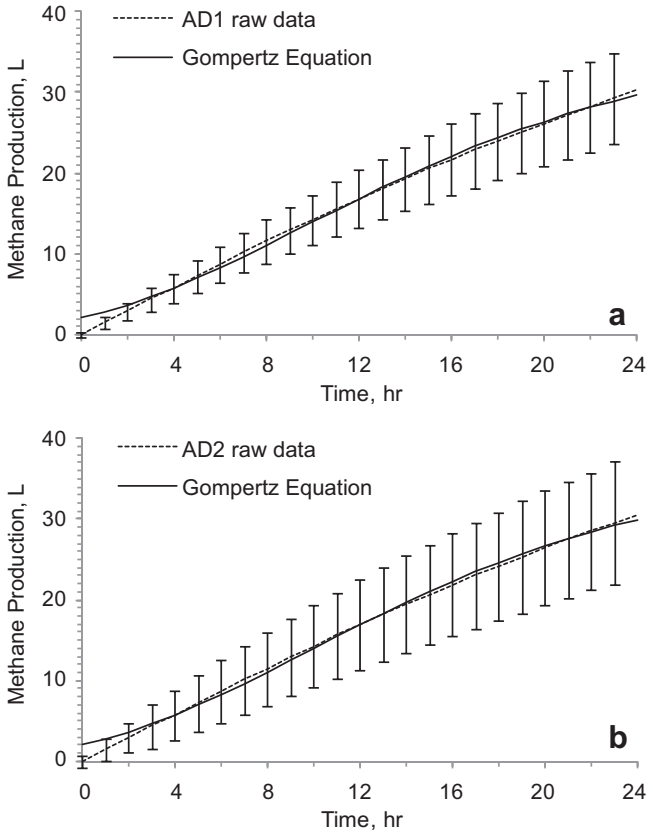

Fig. 2. Methane production over a 24-h operational cycle (average and standard deviation (n = 10)) for: (a) AD1, and (b) AD2. Pertinent statistics are presented in Table 2. Also shown is a curve fit for the Gompertz three-parameter model.

the two operational scenarios, while the calculated volumetric methane yield – estimated at 0.7–0.71 L (L day)$^{-1}$ – would appear to have been identical between the two digesters, since mean biogas production was statistically identical, the AD2 configuration actually produced slightly more methane.

Examining process potential over an operational cycle, as shown (Fig. 2) biogas generation for both AD configurations was generally constant over the entire cycle (i.e., no abrupt changes in the rate of production). The data shown represents 10 discrete cycles within a 20-day period. For the AD2 operational scenario, the comparable process stability and relatively uniform biogas production over a cycle was not necessarily expected, given the partially biodegraded manure substrate. Rather, with the comparatively larger quantity of VFAs supplied from the fermenter to AD2 (Table 2), a higher initial rate of biogas production was expected followed by a reduced rate associated with the less biodegradable solid manure substrate. However, the fermentative and methanogenic consortium clearly established an equilibrium that equated to relatively constant biogas production. Further contributing to this outcome was the relative acetate (HAc)-to-propionate (HPr)

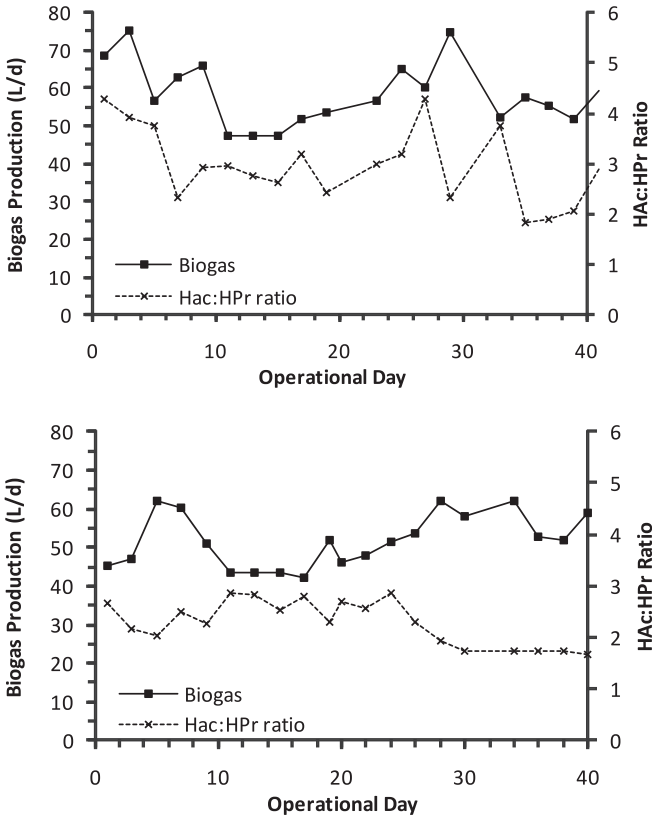

Fig. 3. Biogas production and influent acetate (HAc)-to-propionate (HPr) ratio for: (a) AD1, and (b) AD2 for a 40 days operational window within the 85 operational cycle.

ratio in the AD substrate (Fig. 3); the respective HAc:HPr ratios for AD1 and AD2 were 3.0 ± 0.8 and 2.3 ± 0.4. While acetate is directly converted to methane, propionate must first be oxidized to acetate (via interspecies hydrogen transfer). Thus, the increased quantity of propionate fed to AD2 coupled with the additional time required to oxidize this substrate and the generation of hydrogen (another methane precursor) appeared to enhance temporal process stability in AD2. In terms of modeling the observed biogas rate of production, it has been suggested that the modified three-parameter Gompertz bacterial growth curve model (Zwietering et al., 1990) could represent AD methane synthesis (Behera et al., 2010). Applying this theory, the Gompertz equation was curve fit (minimizing the sum of squared errors) to the AD1 and AD2 data. Modeling results (Fig. 2a and b) would suggest that the respective AD microbial consortia were metabolically maintained in the middle-to-late log-growth phase. The resulting Gompertz parameters were as follows (AD1 and AD2, respectively): P (CH_4 production potential; 36.7 and 37.1 L CH_4), R_{max} (CH_4 production rate; 1.46 and 1.48 L CH_4 h^{-1}), and λ (lag-phase time; 0.46 and 0.58 h). Considering the goal of maximizing value recovery from manure, these results are encouraging in that it would appear that the two-stage AD configuration was semi-optimized.

The observed gross biogas yield metrics were generally comparable between AD1 and AD2 (Table 2), although in most cases slightly lower for AD2. The biogas yield values for both ADs were also generally comparable to that observed by others (Borole et al., 2006; Karim et al., 2005; Khanal, 2008; Rico et al., 2011; Yilmaz and Demirer, 2008). In particular, in feeding a mesophilic AD (1500 L; OLR of 2.0 g VS (L day)$^{-1}$; fed on 30 min intervals) with dairy manure supernatant from a screw press, Rico et al. (2011) observed biogas yields at an RT of 20 days (i.e., same as AD1) and 16.7 days (i.e., effectively the same as AD2) of 0.327 and 0.336 L (g VS applied)$^{-1}$, and methane yields of 0.206 and 0.219 L CH_4 (g VS applied)$^{-1}$, respectively. While their OLR was lower than applied in this study, the respective yields were comparable. However, their influent VFA concentration was 2–5 times higher than realized in this study's ADs, which certainly would have their enhanced biogas production given the high quality methane precursors. In contrast, Borole et al. (2006), operating a 96 L mesophilic AD fed at a comparable OLR to this study, reported biogas and methane yields of 0.64 and 0.4 L (L day)$^{-1}$, which were markedly lower than observed herein. A probable cause for reduced yields was an imposed operational cycle of 2 days, as compared to the 1 day cycle for this study. Finally, from a modeling perspective Hill developed a simplified steady state mesophilic AD model (Hill, 1991) for estimating methane productivity. Applying this model to AD1 and AD2 and further utilizing an OLR based on HRT (per Hill; note that Hill's OLR is calculated differently than presented in this study), the model would predict 0.86 and 0.17 L CH_4 (L day)$^{-1}$, respectively. While the model estimate slightly overestimated that observed for AD1 (0.7 L CH_4 (L day)$^{-1}$), the model significantly underestimated methane production in AD2 (0.71 L CH_4 (L day)$^{-1}$). Hill's empirical equation predicts a linearly increasing rate of methane production based on AD organic loading rate, and integrates a stress function to model the adverse effects of excess organic loading on biogas production. While others have similarly observed that Hill's model is generally accurate for raw manure waste streams (Karim et al., 2005, 2007), it would appear that the model is not representative for pre-fermented manure. Specifically, Hill's "stress index" parameter does not factor in the effects of a less readily biodegradable organic matter on AD productivity.

3.2. Volatile solids loading/destruction assessment

As described, the two ADs were designed to operate at an OLR of 3.6 g VS (L day)$^{-1}$. However, based on actual average influent VS (Table 2), applied OLRs were 3.7 and 3.47 g VS (L day)$^{-1}$ for AD1 and AD2, respectively. The slight deviations from the design OLR reflect the real variability that is experienced in using manure, which is inherently a heterogeneous substrate; similar variability would certainly occur under full-scale operations. Beyond the system OLR, and as noted above, the actual OLR realized in AD2 (excluding the fermenter) was 4.2 g VS (L day)$^{-1}$; the observed increase in OLR into AD2 was due principally to low VS reduction in the fermenter (Fig. 4), which is consistent with previous fermentation investigations (Coats et al., 2011). While it could be suggested that the higher AD2 OLR should have enhanced biogas production

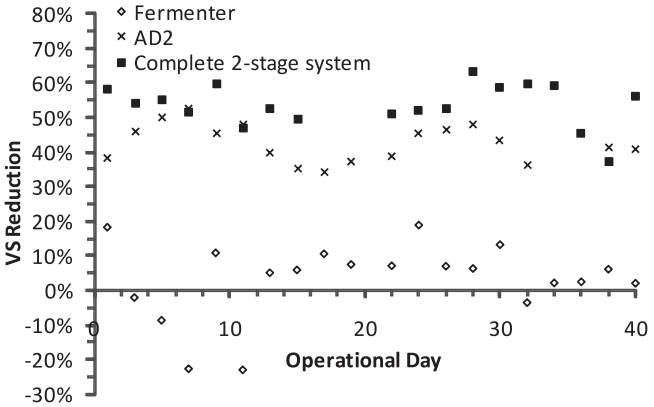

Fig. 4. Volatile solids (VS) destruction for the fermenter and AD2 that comprised the two-stage configuration, for a 40 days operational window within the 85 operational cycle. Also shown is the VS destruction for the combined system.

over that observed in AD1, as detailed above the two systems instead generated statistically comparable biogas volumes. In fact, the gross biogas yield based on VS applied and VS destruction for AD2 was slightly lower than observed in AD1 (Table 2).

Observed VS reduction in the respective digesters is shown in Fig. 5. As would be expected, biogas production increased with higher VS reduction and vice versa. The variable VS reduction was most likely a result of the heterogeneous substrate. Although AD2 was organically loaded at a slightly higher rate than AD1, statistically the VS reduction realized in AD2 was lower ($p = 0.14$). While observed VS reduction was consistent with typical dairy manure AD performance (El-Mashad et al., 2008; Kaparaju and Rintala, 2011), the decreased VS reduction at the higher OLR in AD2 might seem unexpected. Upon further consideration of the respective AD scenarios, however, two aspects likely led to this outcome. First, AD2 was ultimately fed a lower quality (i.e., pre-fermented) substrate. Alone, dairy manure is a moderately difficult substrate to biodegrade, given its relatively high lignocellulosic content; amongst a wide array of organic waste substrates dairy manure exhibits the lowest biodegradability (Ward et al., 2008). Pre-fermentation and the diversion of the VFA-rich substrate further reduced the quantity of readily bio-available carbohydrates for methane production. Note that the fermenter was also operated under semi-optimal conditions for maximum conversion of manure to VFAs (Coats et al., 2011). While this was a known part of the experimental design, the relative impact must be highlighted. A second cause of lower VS reduction in AD2 was the reduced operational RT. AD2 alone (excluding the fermenter) was operated at an RT of 16 days, while AD1 was operated at 20 days. RT has been shown to be a critical factor in VS reduction and biogas production (Khanal, 2008; Speece, 2008).

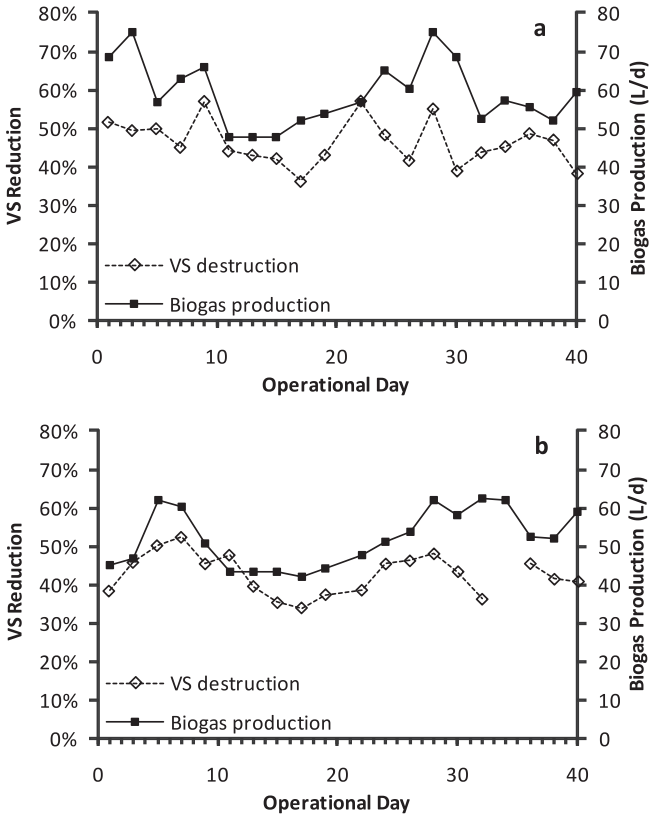

Fig. 5. Volatile solids destruction and biogas production over the operational analysis period for: (a) AD1, and (b) AD2.

3.3. Relative influent VFA contribution to biogas production

In considering the statistically comparable biogas production between AD2 and AD1, while it could be suggested that the successful methane production observed in AD2 was largely a product of significant quantities of ideal substrate (VFAs) being supplied from the fermenter (Table 2), in reality this process variable likely only contributed a small fraction toward biogas production. From a purely stoichiometric perspective, theoretically the AD of 1 g acetate (as COD) would generate 0.35 L of methane at standard temperature and pressure (Gujer and Zehnder, 1983). Applying this stoichiometric ratio, and assuming the best-case scenario that all influent VFAs are acetate, the soluble substrate fed to AD1 and AD2 would have generated, on average, a theoretical maximum of 1.8 L (6.4% of total production) and 5.0 L (18% of total production) of methane per day, respectively. However, considering AD thermodynamics and that the VFAs were not exclusively acetate, actual yield from the influent VFAs would have been less than theoretical. Further, the diversion of VFAs from AD2 (i.e., 2.5 L of VFA-rich fermenter liquor) would similarly be equivalent to a production loss of approximately 20%. Therefore, the loss of methane potential was not significant.

3.4. Assessment of archaeal and methanogenic populations

To complement the AD functional assessment and gain some related perspective on the microbial population structure, qPCR was performed on extracted genomic DNA from five samples over the 85 days operational analysis period. qPCR is a molecular tool that can be applied to compare the relative quantity of microorganisms within and between samples. Quantification is based on the Cq parameter (quantification cycle (Bustin et al., 2009)), which represents the threshold concentration of amplified DNA that can be detected fluorescently by the qPCR instrument; the higher the Cq value, the less initial template material in the sample (i.e., less abundant microbial population). qPCR was applied to comparatively assess the relative fraction of archaea and methanogens enriched in the ADs. Methane is synthesized in anaerobic digesters by hydrogenotrophic and acetoclastic methanogens, of which there are four distinct orders. Methanogens of the hydrogenotrophic orders (i.e., *Methanococcales* (MCC), *Methanobacteriales* (MBT), *Methanomicrobiales* (MMB)), which use H_2 and CO_2 for CH_4 synthesis, are broadly recognized as being the most diverse in regards to species, principally due to favorable bioenergetics (Speece, 2008). Conversely, acetoclastic methanogens use acetate to produce methane, and are combined into a single order (*Methanosarcinales* (MSL)) that has been further subdivided into two principle families (*Methanosarcinaceae* (Msc), and *Methanosaetaceae* (Mst)) (Khanal, 2008). The microbial populations were quantified using 16S rDNA oligonucleotide primers developed by Yu et al. (2005). A principle challenge in performing qPCR on broader-scale microbial orders and families is determination of the 16S rDNA copy number, which is a core parameter in establishing relative quantities of microorganisms in a sample (Pfaffl, 2001). For this study, the respective copy numbers were obtained from the ribosomal RNA operon database. While this database contains a limited number of the methanogenic species targeted in this study, it was nonetheless the best source of rrn data available.

The qPCR operational data and associated results are summarized in Tables 1 and 3. As shown, both ADs were highly enriched by the family Msc, with the AD2 consortium much more so than the AD1 consortium. Conversely, neither AD was significantly enriched with hydrogenotrophic methanogens, although order MMB was more significantly present than order MBT. The enhanced enrichment of Msc in AD2 as compared to AD1 likely contributed to the successful and stable operations detailed herein.

Table 3
Relative quantities of hydrogenotrophic and acetoclastic methanogens (as a fraction of the total archaea population) in AD1 and AD2, as determined using quantitative polymerase chain reaction (qPCR) to amplify 16S rDNA genes (Yu et al., 2005). Also shown are the relative quantities of archaea as a fraction of the total prokaryotic community. Abbreviations as follows: n.d. = non detect, *Methanococcales* (MCC), *Methanobacteriales* (MBT), *Methanomicrobiales* (MMB), *Methanosarcinaceae* (Msc), *Methanosaetaceae* (Mst).

Target group	AD1 relative quantity (%)					AD2 relative quantity (%)				
	1/28/11	2/23/11	3/4/11	4/6/11	4/28/11	1/28/11	2/23/11	3/4/11	4/6/11	4/28/11
MCC	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MBT	1.52 ± 0.11 (n = 3)	0.3 ± 0.04 (n = 3)	0.56 ± 0.03 (n = 3)	0.35 ± 0.12 (n = 3)	1.01 ± 0.67 (n = 3)	0.72 ± 0.08 (n = 3)	0.39 ± 0.13 (n = 3)	0.73 ± 0.03 (n = 3)	0.50 ± 0.07 (n = 3)	0.61 ± 0.36 (n = 6)
MMB	2.22 ± 0.05 (n = 3)	2.72 ± 0.16 (n = 3)	5.52 ± 0.42 (n = 3)	4.27 ± 1.16 (n = 3)	12.55 ± 0.88 (n = 6)	1.42 ± 0.1 (n = 3)	2.80 ± 0.29 (n = 3)	4.19 ± 0.16 (n = 3)	4.13 ± 1.34 (n = 3)	3.35 ± 0.30 (n = 6)
Msc	47.9 ± 1.9 (n = 3)	27.46 ± 1.58 (n = 3)	21.73 ± 0.21 (n = 3)	12.21 ± 1.65 (n = 3)	27.03 ± 6.46 (n = 6)	70.47 ± 1.11 (n = 3)	99.64 ± 6.71 (n = 3)	85.80 ± 1.10 (n = 3)	73.57 ± 4.17 (n = 3)	83.42 ± 11.48 (n = 6)
Mst	0.08 ± 0.01 (n = 3)	0.08 ± 0.01 (n = 3)	0.04 ± 0.01 (n = 3)	0.02 ± 0.01 (n = 3)	0.52 ± 0.05 (n = 3)	0.05 ± 0.01 (n = 3)	0.12 ± 0.01 (n = 3)	0.05 ± 0.01 (n = 3)	0.05 ± 0.01 (n = 3)	0.20 ± 0.02 (n = 3)
Archaea	18.8 ± 0.51 (n = 3)	22.52 ± 2.54 (n = 3)	11.02 ± 1.16 (n = 3)	26.63 ± 1.64 (n = 3)	1.49 ± 0.31 (n = 6)	26.71 ± 2.31 (n = 3)	15.3 ± 3.38 (n = 3)	19.79 ± 1.23 (n = 3)	30.85 ± 1.67 (n = 3)	5.41 ± 0.75 (n = 6)

Table 4
Summary of anaerobic digester carbon balance analysis. Influent solids and volatile fatty acids (VFAs), effluent solids and VFAs, and biogas (CH_4 and CO_2) were included in the analysis.

	AD1			AD2		
	Average (g C)	Standard deviation (g C)	n	Average (g C)	Standard deviation (g C)	n
Influent solids	73.2	7.5	25	87.5	8.6	25
Influent VFAs	1.9	0.7	25	4.8	2.0	25
Effluent solids	42.4	3.5	25	52.5	5.8	25
Effluent VFAs	0.6	0.4	25	0.9	0.7	25
Biogas (CH_4 + CO_2)	31.8	4.0	21	29.0	4.4	21
Balance	0.3 0.7%			9.9 10.7%		

Regarding the acetoclastic methanogens, from a kinetic perspective Msc exhibits an enhanced reproductive capability as compared to Mst (Speece, 2008); combined with the high VFA concentrations in the respective ADs (Table 2) and also the HAc:HPr ratios, which would favor acetoclastic methanogen proliferation, it is no surprise the both ADs were more enriched in Msc over Mst. Integrating the observed microbial structural data with AD function, as shown (Fig. 1a and b) there appeared to be a correlating trend between relative Msc fraction and total biogas synthesized. In fact, the data indicates that total biogas production in both ADs was closely linked with the Msc population. Considering more broadly the total archaeal population, across the 85 days analysis period the ADs were comparably enriched in archaea (Table 3). However, the relative archaeal fractions were generally higher than the "typical" estimated maximum of 10% of the total prokaryotic population (Garcia et al., 2000). The advanced molecular techniques applied in this study likely explain the disparity, as these "typical" values were estimated using less sophisticated microbiological methods. Finally, contrasting the archaeal data with the Msc and biogas data (Fig. 1a and b), it would appear that as the Msc fraction (of total archaea) decreased the archaeal fraction (of total prokaryotes) increased slightly, and vice versa. Rather than indicating a significant increase in the non-Msc archaeal population, recognizing that the Msc population demands acetate for biogas production, these results suggest instead that the fermenting bacterial population (which provides the precursor acetate) experienced some variability over time. A higher fermenting microbial population would, (1) decrease the relative archaeal fraction in the ADs (since archaea was determined as a fraction of prokaryotes, which includes the fermenters), but (2) increase the Msc fraction of total archaea, since additional acetate would be available for Msc growth.

3.5. Carbon mass balance analysis

A carbon mass balance was developed for both ADs as a complement to the performance analyses. The mass balance analysis included total solids, VFAs, methane, and carbon dioxide. VFAs and biogas quantities were converted to a carbon basis using molecular weights, while total solids were converted based on TOC analysis. For both ADs, the dominant components in the carbon mass balance were influent and effluent solids, followed by biogas quantities (Table 4). Conversely, influent and effluent VFAs were a nominal fraction of the total carbon influent/effluent flux. As shown, the carbon mass balance closed almost completely for AD1. However, the same cannot be said for AD2; specifically, approximately 10% of the carbon fed to AD2 is unaccounted for in the effluent emissions. The 10% un-accounted for carbon could have been present as compounds not analyzed, principally long chain fatty acids (LCFAs). In particular, the pre-fermented manure likely contained more lipids (fractionally), which would be hydrolyzed to LCFAs; the shorter RT in AD2 may have been insufficient to fully oxidize the LCFAs to short chain VFAs. Considering the larger scale of these ADs, small operational variability in influent/effluent volumes could also have contributed to this relatively small imbalance in carbon.

4. Conclusions

The purpose of this study was to demonstrate the viability of a novel two-stage AD process receiving pre-fermented dairy manure. In summary, it was observed that AD of thickened pre-fermented manure can generate comparable biogas quantities to AD using raw manure, with enhanced methane content. Further, AD stability

using pre-fermented manure was enhanced over that processing raw manure. The two-stage AD was more enriched with the acetoclastic methanogen *Methanosarcinaceae* (Msc; compared to AD of raw manure) and biogas production appeared linked with the Msc fraction. In fact, the enhanced enrichment of Msc likely contributed to the successful and stable operations.

Acknowledgements

This material is based on work supported through a grant provided by the Idaho National Laboratory through the Idaho Center for Advanced Energy Studies (CAES). Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the funding agency.

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