Bioresource Technology 151 (2014) 249–257

Contents lists available at ScienceDirect

Bioresource Technology

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

Molecular characterization of anaerobic digester microbial communities identifies microorganisms that correlate to reactor performance

Jennifer R. Town [a,b], Matthew G. Links [a,b], Terrance A. Fonstad [c], Tim J. Dumonceaux [a,b,*]

[a] Agriculture and Agri-Food Canada, 107 Science Pl., Saskatoon, SK S7N 0X2, Canada
[b] Department of Veterinary Microbiology, WCVM, University of Saskatchewan, 52 Campus Dr., Saskatoon, SK S7N 5B4, Canada
[c] Department of Civil and Geological Engineering, University of Saskatchewan, 57 Campus Dr., Saskatoon, SK S7N 5A9, Canada

HIGHLIGHTS

- Digesters with the highest methane accumulation were efficient at catabolizing VFA.
- The abundance of individual organisms correlated to reactor performance.
- The same microorganisms dominated the reactors regardless of input material.
- Both hydrogenotrophic and acetoclastic methanogens were detected.
- Hydrogenotrophic methanogenesis was the dominant methane-producing pathway.

ARTICLE INFO

Article history:
Received 16 August 2013
Received in revised form 10 October 2013
Accepted 15 October 2013
Available online 30 October 2013

Keywords:
Anaerobic digestion
Pyrosequencing
Methanogenesis
Syntrophic acetate oxidation

ABSTRACT

A time-course analysis was conducted of thermophilic anaerobic digestion of dairy manure and wheat distillery thin stillage. Sequencing of chaperonin targets provided a phylogenetic survey of both bacteria and archaea in the digestate, along with an appraisal of the diversity of the reactor microbiome. A total of 1129 bacterial operational taxonomic units (OTU) were detected in the reactors, with OTU related to *Clostridium* becoming numerically dominant by day 7, and *Acetivibrio*-related OTU by day 35. Archaeal communities were less diverse, with 19 OTU detected representing both acetoclastic and hydrogenotrophic methanogens. Regardless of input material, the same organisms came to dominate the reactors, reflecting strong selective pressures present in the digesters. Principal coordinate analysis of the microbial communities showed that the bacterial communities clustered based on factors other than input material. Bacterial and archaeal OTU were identified with significant correlations to performance parameters, suggesting important roles in the methane production pathway.

1. Introduction

If sufficiently optimized, anaerobic digestion (AD) of agricultural waste is an efficient waste disposal system and a source of renewable energy. The process is dependent on the metabolic activity of a complex microbial consortium to convert the input material, which may consist of agricultural, animal or food processing waste, into methane gas. Optimizing the operation conditions to favor the growth and metabolic activity of organisms that break down the organic input material to produce specific end products is desirable; however, a lack of detailed understanding of these microbial communities has hindered progress in this regard (Dar et al., 2008). The current practice of modifying organic loading rates or altering the pH of the digestate has yielded mixed results (Werner et al., 2011; Westerholm, 2012). Moreover, a lack of tools for directly monitoring the composition of the digester microbiome further complicates the situation with drops in methane production and spikes in volatile fatty acid (VFA) accumulation often going unexplained (Ward et al., 2008).

Although most AD is maintained at mesophilic temperatures, thermophilic conditions provide the most thorough breakdown of the organic inputs. When sufficiently optimized thermophilic AD is more efficient and requires shorter hydraulic retention times; however, the thermophilic microbial community has been shown to be less diverse, more unstable, and more sensitive to fluctuations in operational parameters (Weiland, 2010). Grain ethanol distillery waste products, such as those generated from corn, wheat, and barley, can be converted to methane under thermophilic conditions, which provides a source of relatively stable and nutrient rich organic material that might otherwise be a waste

* Corresponding author at: Agriculture and Agri-Food Canada, 107 Science Pl., Saskatoon, SK S7N 0X2, Canada. Tel.: +1 306 956 7653; fax: +1 306 956 7247.
E-mail address: tim.dumonceaux@agr.gc.ca (T.J. Dumonceaux).

0960-8524/$ - see front matter
http://dx.doi.org/10.1016/j.biortech.2013.10.070

product (Ziganshin et al., 2011). The distillation process during ethanol production consumes essentially all of the available six carbon sugars in the stillage, converting them to ethanol using yeast fermentation. The remaining stillage waste material contains predominantly five carbon sugars, complex carbohydrates such as cellulose, lipids and proteins (Mustafa et al., 2000). Studies examining the biogas potential of this substrate have shown the process to be energy efficient in terms of carbon balance, especially when the energy produced from the digester is used to offset energy expenditures during ethanol production (Agler et al., 2008; Eskicioglu et al., 2011; Schaefer and Sung, 2008). Recently, studies have examined the co-digestion of stillage waste with manure as a way to boost methane production as well as increase the stability and consistency of the AD process (Westerholm, 2012).

Previous studies examining the composition and dynamics of the bacterial communities associated with thermophilic AD reactors have left many unanswered questions. While microbial communities appear to undergo large shifts in species diversity over the short term, they show surprising robustness and consistency over the long term, even after changes in operating parameters or exposure to toxins (Schauer-Gimenez et al., 2010; Werner et al., 2011). Many of these studies have been unable to show a consistent relationship between microbial composition and digester performance, in particular methane production and volatile solids consumption (Krause et al., 2008; Liu et al., 2009; Wang et al., 2009). More recently, molecular characterization of digester communities combined with quantitative PCR assays have successfully correlated specific microorganisms to digester performance parameters including methane production and volatile fatty acid catabolization (Lv et al., 2013).

During AD, organic material is converted to methane by a microbial consortium consisting of both bacteria and methanogenic archaea. Anaerobic bacteria initially degrade the substrate by hydrolysis and acidogenesis. The end products of this initial breakdown are CO_2, H_2 and VFA including acetate, propionate, butyrate, formate, succinate and lactate. Acetogenic bacteria further oxidize the VFA, generating acetate, CO_2 and H_2. The final stage, methanogenesis, is achieved through the metabolic activity of acetoclastic or hydrogenotrophic methanogens. Acetate can be converted to CH_4 directly by acetoclastic methanogens, of which *Methanosarcina* and *Methanosaeta* are the most frequently described (Demirel and Scherer, 2008). Alternatively, acetate can be oxidized to CO_2 and H_2 by bacteria in a syntrophic association with hydrogenotrophic methanogens (e.g. *Methanothermobacter*, *Methanoculleus*) (Demirel and Scherer, 2008). To achieve optimal methane production in this system, balance must be maintained between bacterial and archaeal metabolic activity. An increase in metabolic intermediates can be inhibitory to other critical organisms in the consortium, and result in reduced reactor performance or a complete collapse of methanogenesis. A better understanding of the specific microorganisms that are essential at each stage of methanogenesis and their interaction with each other is critical for optimizing reactor design and operation as well as troubleshooting issues with regard to reactor performance.

Molecular methods, including universal target amplification combined with pyrosequencing and quantitative PCR, allow analysis of the microbial community at a resolution that can distinguish between closely related species, and at a depth that permits detailed examination of community structure parameters such as richness and diversity. A protein coding gene, chaperonin 60 (*cpn*60) is universally conserved among eukaryotes, bacteria and some archaea and, while there are exceptions, is more commonly present as a single copy gene, allowing for accurate quantification of organisms (Hill et al., 2004). Type I chaperonins (*cpn60*) are present in bacteria and some archaea, have been shown to provide greater resolution between closely related organisms compared to 16S rRNA-encoding genes, and have recently been proposed as a suitable molecular barcode for bacteria using the International Barcode of Life criteria (Links et al., 2012). Type II chaperonins, or thermosomes, are present in archaea and the eukaryotic cytosol and universal primers have recently been developed for amplifying this target from mixed microbial communities (Chaban and Hill, 2012). A database of reference type I and II chaperonin sequences (www.cpndb.ca) provides a breadth of reference sequences on par with that available for 16S rRNA-encoding sequences (Hill et al., 2004). These tools have been exploited to examine microbial communities from a variety of environments (Chaban and Hill, 2012; Dumonceaux et al., 2006), but no previous studies have examined both type I and type II chaperonins in mixed bacterial/archaeal communities such as those associated with AD.

A time-course analysis was performed of bacterial and archaeal communities within thermophilic digesters processing wheat ethanol stillage and dairy cattle manure, and molecular methods were used to quantify and monitor organisms critical in the methanogenesis pathway. Ecological parameters of the microbial communities were examined (evenness, richness, and diversity), as these have been shown previously to affect reactor performance, especially as it relates to reactor variability (Schauer-Gimenez et al., 2010; Werner et al., 2011). The information gained by characterizing the microbiome of both high- and low-performing digesters will help to identify a target microbial population and composition associated with maximum reactor performance. The data can also be used to inform reactor design and dictate the operational parameters for introducing and recycling microorganisms during digestion.

2. Methods

2.1. Input materials

Wheat grain distillery thin stillage was obtained from Terra Grains Inc. (Moose Jaw, SK, Canada), a facility producing ethanol from dry-ground wheat grain. Manure was collected from dairy cattle (University of Saskatchewan, Saskatoon, SK, Canada). The starter inoculum (INC) was generated by incubating dairy cattle manure anaerobically at 55 °C for 2 weeks prior to beginning the trial. Total (TS) and volatile (VS) solids for each of the input materials were determined using standard protocols (American Public Health Association, 1995). Values for %VS and %TS of the input material are listed in Supplemental Table S1.

2.2. Bench-scale AD

Bench-scale thermophilic reactors digesting inoculum (INC), dairy manure (MAN), wheat grain distillery thin stillage waste (TST) or thin stillage combined with dairy cattle manure (TSM) were set up in 1 L glass bottles and sealed with air-tight lids fitted with silicone septae. Dairy manure or thin stillage were added individually or mixed in a 1:1 ratio of stillage:manure based on VS content. The stillage or stillage/manure combinations were then mixed with a starter inoculum in a 1:1 ratio based on VS content. The total volume of material in each digester was adjusted to 300 mL and ~5% TS with sterile water. All input combinations were run in duplicate. The initial pH of the digestate mixtures ranged from 7.4 to 9.3. Bottles were sealed and flushed with N_2 gas at 82.74 kPa (12 psi) for 5 min using an outlet needle before being bled down to 3.45 kPa (0.5 psi), and incubated at 55 °C for 48 days in an anaerobic chamber with an atmosphere of 85% N_2, 10% H_2 and 5% CO_2.

2.3. Sample collection

Biogas accumulation in the digesters was monitored at 55 °C every 2–3 days using a pressure transducer (Sper Scientific, Scottsdale, AZ, USA) equipped with a 25G needle, and volume measurements were calculated at standard temperature and pressure. Gas samples were taken, as needed, when reactor pressure exceeded 34.5 kPa (5 psi). Gas samples were extracted using a 20 mL syringe equipped with a stopcock (Cole Parmer, Vernon Hills, IL, USA) and 25G needle and immediately transferred to a 5 mL evacuated, dehumidified vial and stored at 4 °C until analysis. Following gas sample extraction, digestate samples were collected by removing the reactor lid within an anaerobic chamber and sampling 3 mL of digestate using a wide-bore pipette. Samples were stored at −80 °C until DNA extraction. Reactors were then flushed with N_2 gas at 82.74 kPa (12 psi) for 5 min using an outlet needle before being bled down to 3.45 kPa (0.5 psi) and returned to the incubator.

2.4. Biogas analysis

The relative percentage of CO_2 in each gas sample was quantified using a Varian Micro-GC (CP-2003, Agilent, Santa Clara, CA, USA) equipped with a 10 m Poraplot U column and thermal conductivity detector (TCD). Relative percentages of O_2, N_2, and CH_4 were quantified using a 10 m molecular sieve column and TCD. Injector and column temperatures were 110 °C and 100 °C respectively and the equipment was calibrated using certified standards.

2.5. Substrate analysis

Digestate samples taken as described above were diluted 1:5 in water, centrifuged, and loaded onto an HPLC (#1515, Waters, Milford, MA, USA) equipped with an Aminex HPX-87H column (Biorad, Hercules, CA, USA). Sample peaks were identified and quantified by comparison to standard curves for formate, succinate, lactate, acetate, propionate, and butyrate.

2.6. Universal target amplification and sequencing

Total genomic DNA was extracted from end product digestate samples using a modified bead beating method as described previously (Dumonceaux et al., 2006). The *cpn*60 (type I chaperonin) and thermosome (type II chaperonin) universal targets were amplified using multiplex identifier (MID) tagged primer cocktails and amplification conditions previously shown to maximize the range of organisms detected (Chaban and Hill, 2012; Hill et al., 2005). All primer sequences are listed in Supplemental Table 2. For amplification of the type I *cpn*60 target, each reaction contained: 1X PCR buffer (Invitrogen, Carlsbad, CA, USA), 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 100 nM each of H279/H280, 300 nM each of H1612/H1613 and 1U of Platinum Taq (Invitrogen, Carlsbad, CA, USA) and cycling conditions of 1× 95 °C, 5 min; 40× 95 °C 30 s, 42–60 °C 30 s, 72 °C 30 s; 1× 72 °C 2 min. Type II chaperonins were amplified under the same conditions using 437.5 nM each of JH0175/JH178 and 62.5 nM each of JH0268/JH0269 and cycling conditions of 1× 95 °C, 5 min; 40× 95 °C 30 s, 55 °C 30 s, 72 °C 30 s; 1× 72 °C 2 min. Amplification products from replicates for each digester condition were pooled and then sequenced using the Roche 454 GS FLX with Titanium chemistry (Branford, CT, USA).

2.7. Pyrosequencing data analysis

Sequencing reads were processed using mPUMA (Links et al., 2013) with assembly being performed using Trinity with an Inchworm kmer size of 31 bp. OTU-frequency data was calculated using a data set of pyrosequencing reads randomly subsampled to the smallest library size; 8500 for the type I libraries and 3500 for the type II libraries. OTU frequency data was used as input for Mothur, Unifrac, and R to correlate OTU abundance, taxonomic composition and microbial community structure with reactor performance data.

2.8. Quantitative PCR assays

OTU-specific primers were designed using Beacon Designer v 7.0 (Premier Biosoft, Palo Alto, CA, USA) such that primer similarity was restricted to the desired OTU, avoiding cross-amplification of other assembled OTU sequences. Primers were validated by amplifying the target from an AD template, cloning the amplicon into pGEM-T Easy vector (Promega, Madison, WI, USA), and sequencing representative clones to verify that only the intended target was amplified. Previously described 16S rRNA-based universal primers for amplifying bacteria (Lee et al., 1996) were used to estimate total bacterial load in the digestate. Novel primers for estimating total archaeal load were designed based on all publically available aligned 16S sequences. Universal archaeal primers were validated by amplifying genomic DNA from a taxonomically diverse range of archaea (Supplemental Fig. S1). Additionally, amplicon generated from digestate with an abundance of both bacteria and archaea was cloned; 85 clones were sequenced, of which 100% were of archaeal origin (Supplemental Table S3), indicating that the primers were sufficiently specific to avoid bacterial cross-amplification. For each qPCR assay, cloned amplicon was used to generate standards from 10^7 to 10^1 gene copies per reaction. Target OTU sequences were amplified using EvaGreen qPCR amplification mix (BioRad, Hercules, CA, USA) with 400 nM of each primer; primer sequences and amplification conditions are listed in Supplemental Table S4. Specific OTU, total Archaea, and total Bacteria abundance estimates were correlated to methane proportion in biogas samples, daily methane production, and acetate accumulation by Spearman's Rank analysis using R ($\alpha < 0.01$).

3. Results and discussion

3.1. Biogas composition

Biogas composition analysis in digesters processing inoculum (INC) and manure (MAN) showed that the proportion of methane in the biogas peaked at 88% and 83% respectively (Fig. 1A). Although the methane proportion in digesters processing thin stillage alone (TST) did reach >80% (Fig. 1A), the daily biogas production trailed those of other inputs (Fig. 1B). The high proportion of methane being produced in these reactors indicates that highly active methanogen populations were present, however the decrease in daily production in comparison to INC and MAN reactors suggests that the breakdown of the input material is likely a rate limiting step when processing stillage waste. By contrast, the digesters processing thin stillage with manure (TSM) reached a maximum of ~50% methane in the reactor headspace, declining to 0% methane by day 31. This suggests poor metabolic activity by methanogenic archaea as a rate limiting factor for methane production in these reactors.

The disparity in performance between the TST and TSM digesters provided an opportunity to examine in more detail the environmental and/or microbiological reasons for the failure of the methanogenesis pathway, as the co-digestion of whole stillage from cereals with manure has previously been shown to have good methane production under similar conditions (Westerholm, 2012).

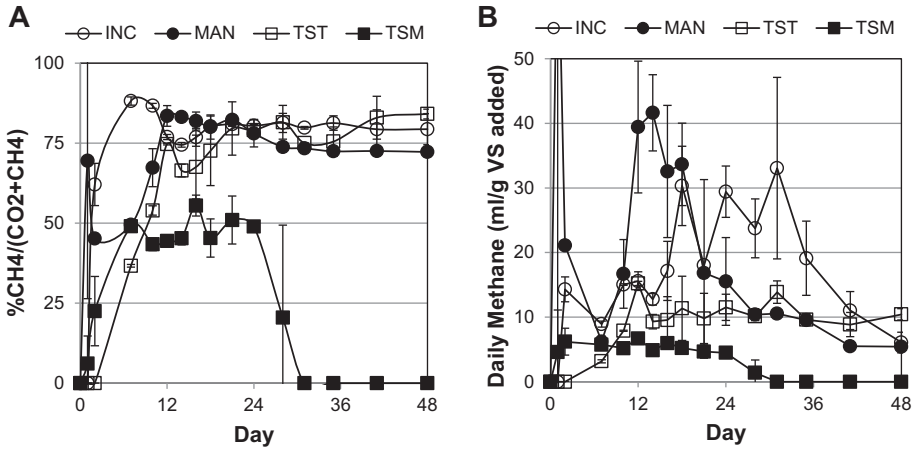


Fig. 1. Average methane proportion (A) and daily production (B) in reactors processing inoculum (INC), manure (MAN), thin stillage (TST) and thin stillage with manure (TSM). Biogas samples were collected from duplicate reactors, transferred to a dehumidified, evacuated vial, and gas composition was determined using gas chromatography.

3.2. VFA metabolism

Acetate was the most abundant VFA in the reactors, with butyrate and propionate produced in lesser quantities (Fig. 2). Digestate pH correlated negatively with VFA accumulation, particularly with acetate accumulation (Pearson correlation = −0.816, p-value < 0.01). Formate, succinate and lactate accumulation was not detectable in all digesters. The MAN reactors were the most efficient at catabolizing VFAs, completely eliminating VFA accumulation in the digestate after day 21. While the early accumulation of VFA was similar for the INC and TST reactors, the TST reactors were much less efficient at converting VFA to methane, resulting in continued VFA accumulation at the end of the trial period. The TSM reactors showed almost no VFA catabolization, resulting in very poor methane production. These data suggest that in the TSM reactors, the initial steps of the methanogenesis pathway (hydrolysis, acidogenesis, and acetogenesis) were successful and the root cause of the performance disparity was the transition from acetogenesis to methanogenesis, which would be presumed to be caused by a dysbiosis in those reactors.

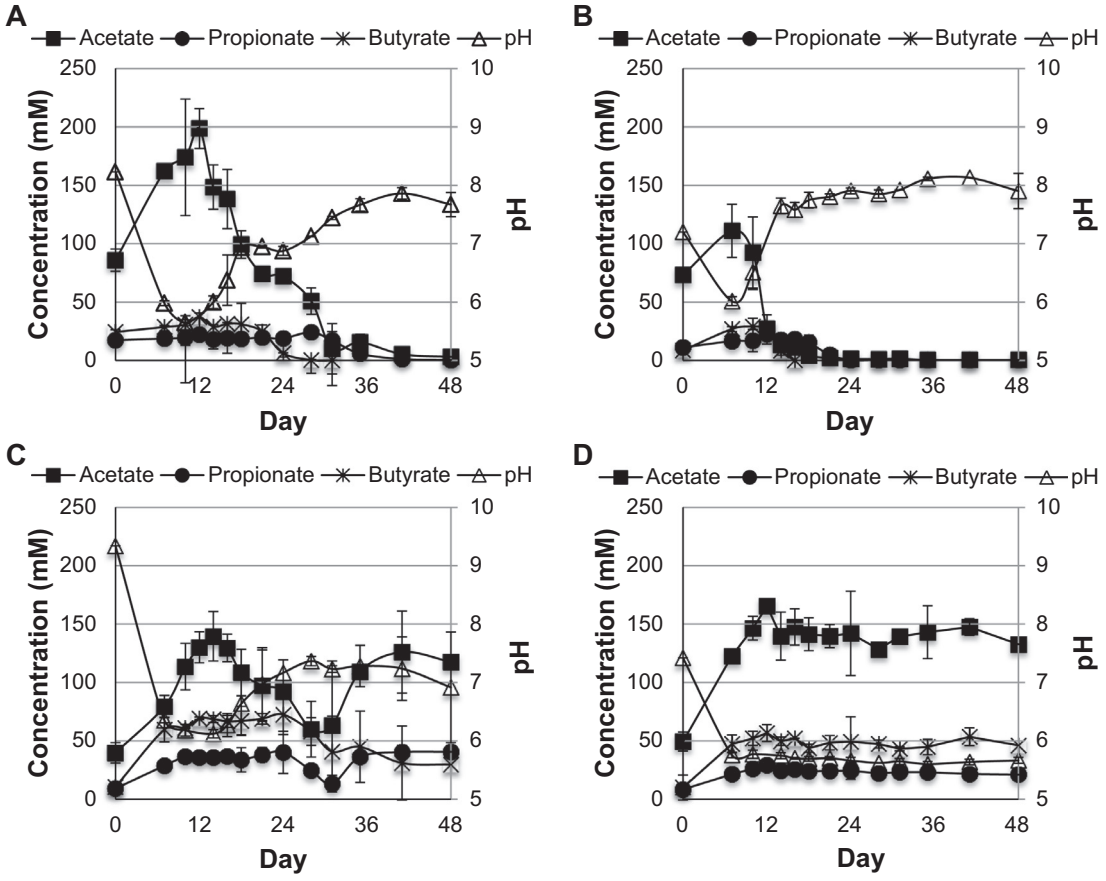


Fig. 2. Average VFA accumulation in digesters processing INC (A), MAN (B), TST (C) and TSM (D). Digestate samples were collected from duplicate reactors, diluted, filtered, and volatile fatty acid concentration was measured using HPLC.

3.3. Microbial community diversity

The addition of manure, a rich source of microorganisms, to the input mixture increased the initial bacterial community richness and evenness as measured by Chao1 and Simpson's indices for the type I (*cpn*60) target (Supplemental Fig. 2). After the first week of incubation, the selective pressure from both the thermophilic environment and the composition of the input material decreased the richness dramatically, particularly in reactors processing thin stillage. Rank-abundance curves showed that the minimum bacterial diversity was reached at day 7 for TST and TSM reactors and day 21 for INC and MAN reactors (Fig. 3). By day 48, all digester inputs showed an increase in diversity resulting from both a decrease in abundance of the most dominant OTU, as well as an increase in the number of different OTU detected in the pyrosequencing libraries. These dynamic changes in community diversity were likely the result of proliferation of organisms that were adapted to the selective pressures in this environment including thermophilic temperatures, high concentrations of volatile fatty acids, and low pH. This pattern of selective microorganism proliferation during anaerobic digestion has been seen previously, with decreasing richness and increasing evenness observed over the course of thermophilic digestion (Gannoun et al., 2013).

The archaeal communities were much less diverse and showed very little change in diversity over the course of the trial as measured by Chao1 and Simpson's indices for the type II chaperonin target (Supplemental Fig. 3).

3.4. Microbial community composition

Bacterial (type I chaperonin) and archaeal (type II chaperonin) pyrosequencing data was assembled into 1129 and 19 OTU respectively. The relative frequencies of all assembled type I *cpn*60 OTU in each digester are shown in Fig. 4. By day 7, the bacterial pyrosequencing libraries were dominated by a single OTU, comprising 39–93% of the total reads regardless of the initial inputs. This OTU, most closely related to *Clostridium leptum* (74% identity), was most abundant in the TST digesters, and is likely critical to the breakdown of the organic material. Other *Clostridium* spp. have been previously associated with thermophilic digestion of stillage waste from rapeseed fermentation (Luo et al., 2011), and several strains isolated from digester sludge processing other waste streams have shown cellulolytic properties (Johnson et al., 1981; Madden, 1983; Sleat et al., 1984), producing significant amounts of hydrogen (Levin et al., 2006). Their abundance in this digester system is consistent with high levels of VFA accumulation and their hypothesized role as contributors to hydrogenotrophic methane production.

Pyrosequencing libraries from the INC digesters remained dominated by *Clostridium* spp. throughout the trial while libraries from the MAN, TST and TSM digesters shifted to a community profile dominated by a single OTU most closely related to *Acetivibrio cellulolyticus* (78% identity) by day 35 (Fig. 4).

The archaeal communities in these digesters showed very little diversity, and the type II chaperonin libraries were dominated by a single OTU that most closely resembled the hydrogenotrophic methanogen *Methanoculleus bourgensis* (90% identity). The second-most prevalent OTU was most closely related to an acetoclastic methanogen, *Methanosarcina barkeri* (88% identity). Together, these two OTU accounted for 95.6–99.9% of the total pyrosequencing reads in the type II chaperonin library (Supplemental Fig. S5; Supplemental Table S5).

Regardless of the composition of the input material or the initial microbial community distribution, the same organisms came to dominate the reactors, reflecting the strong selective pressures present in the thermophilic digestion environment, a phenomenon previously observed in digesters processing agricultural wastes (Ziganshin et al., 2013).

3.5. Unifrac

Principal coordinate analysis (PCoA) of weighted Unifrac values of the type I pyrosequencing data confirmed that bacterial communities clustered based on their point in the methanogenesis pathway as opposed to input material, with 78% of the variability explained by the first two coordinates. Samples from days 7 and 21 were more similar to each other than to days 35 and 48, regardless of input material (Fig. 5). Over the course of the trial, the

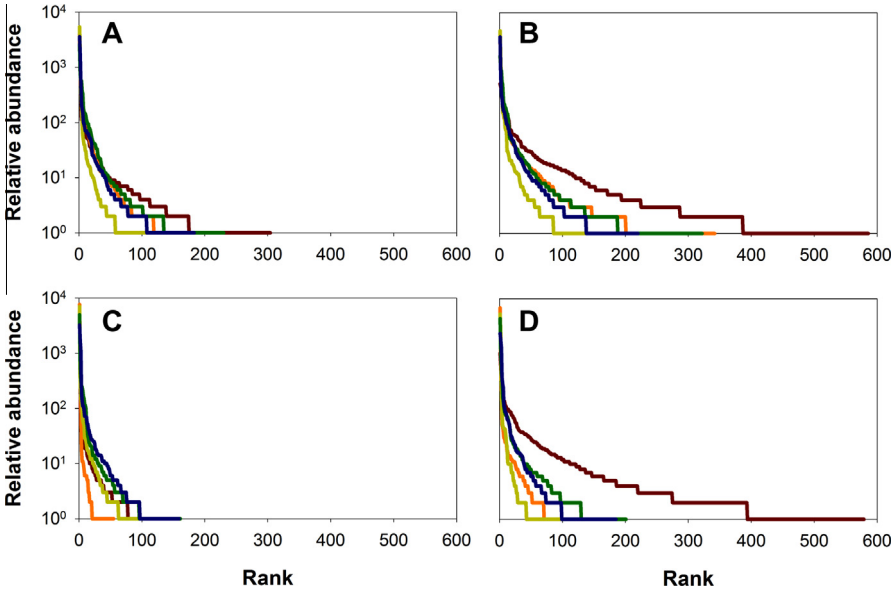


Fig. 3. Rank-abundance curves for OTU frequency in the pyrosequencing libraries from INC (A), MAN (B), TST (C) and TSM (D) digesters, for days 0 (red), 7 (orange), 21 (yellow), 35 (green) and 48 (blue). OTU-frequency data from subsampled pyrosequencing libraries was used to calculate rank-abundance using Mothur.

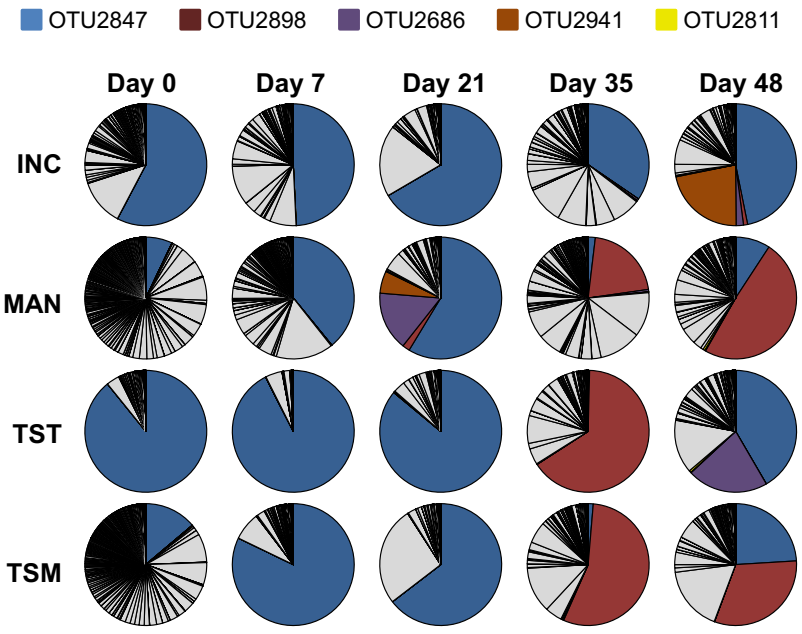
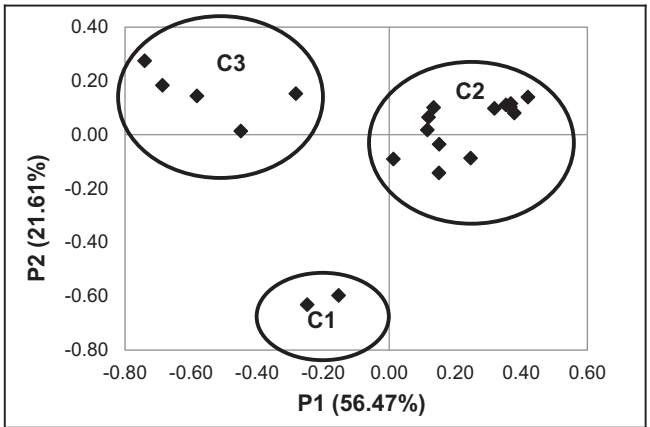


Fig. 4. Relative OTU distribution in the type I chaperonin (*cpn*60) pyrosequencing libraries. Pyrosequencing reads generated using pooled amplicon from duplicate digesters was assembled using mPUMA, and the relative abundance of individual OTU is shown. OTU sequences were compared to the cpnDB reference database for taxonomic identification: OTU2847 (75% *Clostridium leptum*), OTU2898 (78% *Acetivibrio cellulolyticus*), OTU2686 (75% *Clostridium thermocellum*), OTU2941 (74% *Heliobacterium modesticaldum*), and OTU2811 (85% *Clostridium thermocellum*). OTU frequency data to identify OTU with very low abundance is available in Supplemental Table S6.

inoculum-only digesters showed a more consistent community composition compared to the other inputs. Digesters processing either thin stillage, manure or both showed a dramatic shift in bacterial community composition at day 35. This clustering may indicate that environmental conditions such as temperature, pH and VFA concentration had a stronger selective effect on microbial community composition than input material and that these dynamic community shifts may be relatively consistent across reactors containing different types of stillage and other cellulosic wastes. Recognizing that a single community composition could be highly effective for processing a variety of inputs could inform the design of a more robust and universal reactor for anaerobically digesting agricultural wastes.



Treatment	Cluster1	Cluster2	Cluster3
INC		Day 0, 7, 21, 35, 48	
MAN	Day 0	Day 7, 21	Day 35, 48
TST		Day 0, 7, 21, 48	Day 35
TSM	Day 0	Day 7, 21	Day 35, 48

Fig. 5. Weighted Unifrac analysis of digestate bacterial communities. Subsampled OTU-frequency and phylogenetic data was used as input for principal coordinate analysis (PCoA) of weighted Unifrac values.

3.6. Correlation of OTU abundances to reactor performance parameters

The pyrosequencing libraries were used as a starting point for identifying organisms critical to methanogenesis in these reactors. OTU frequency was correlated to reactor performance data using Spearman's Rank correlation ($\alpha < 0.05$), and OTU with significant correlations to performance objectives were subjected to further analysis (Supplemental File S1). OTU2847 and OTU2898 were also selected for further analysis based on their numerical dominance. While these two OTU did not correlate to performance parameters, pyrosequencing analysis uses end-point PCR amplicon, and its limited dynamic range for detecting differences in abundance is exacerbated when organisms are highly abundant. Selected organisms, along with total archaea and bacteria, were quantified in reactor replicates using OTU-specific qPCR assays (Fig. 6), and their abundances were correlated to methane and acetate accumulation at critical time points using Spearman's Rank correlation (Table 1).

3.7. Bacterial OTU (type I chaperonin)

Total bacterial load, as estimated by qPCR (Supplemental Fig. S4), did not correlate significantly to any performance parameter (Table 1), consistent with what has been seen in other studies (Krause et al., 2008; Liu et al., 2009; Wang et al., 2009). This does indicate however that the proliferation of selected organisms within the bacterial community show a strong positive correlation to reactor performance measures including methane production and acetate production and catabolization.

OTU2847, most closely related to *C. leptum* (74% identity) and the most abundant OTU in the pyrosequencing libraries, was initially present at $\sim 10^7$–10^8 genomes/mL digestate, increased

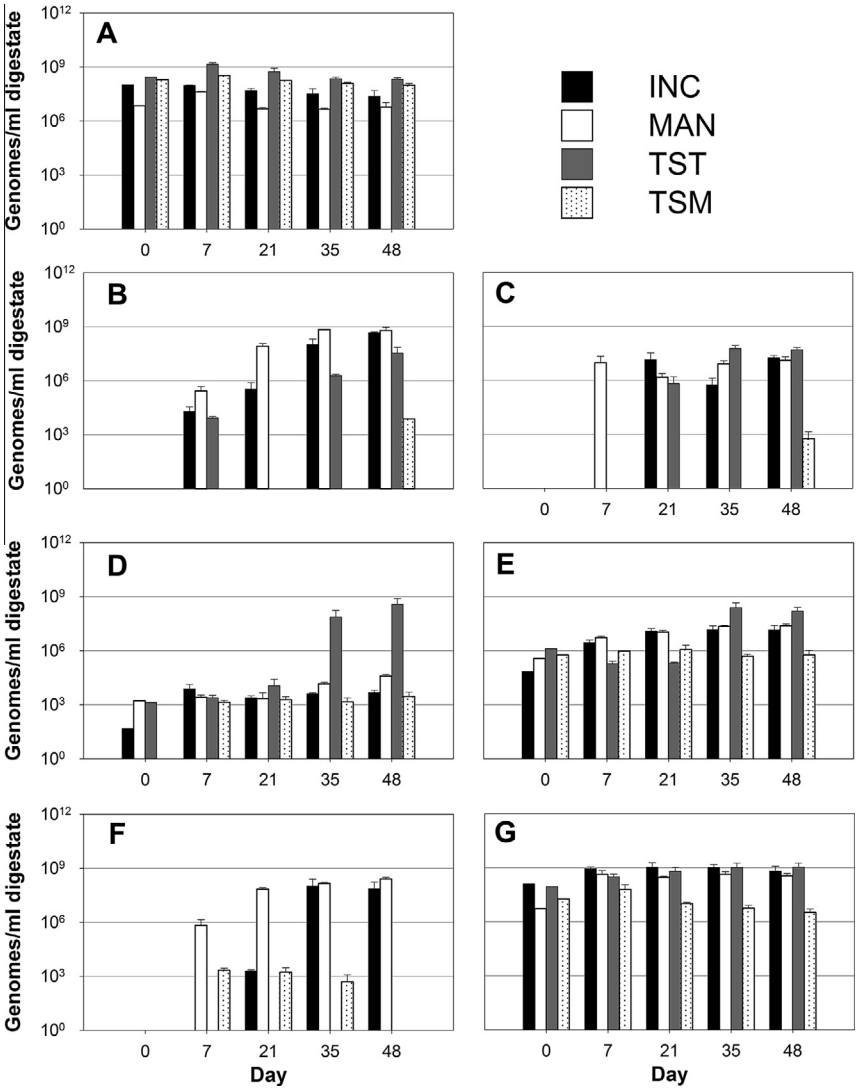


Fig. 6. Average abundance of selected OTU in reactors processing INC, MAN, TST and TSM. OTU-specific quantitative PCR assays were used to quantify selected organisms in digestate from duplicate reactors at five time points during digestion for OTU2847 (A), OTU2898 (B), OTU2811(C), OTU2941(D), OTU2686(E), OTU795(F), and OTU805(G).

Table 1
Spearman's Rank analysis correlating OTU abundance and reactor performance data. OTU abundance, along with total archaea and total bacteria, was estimated in biological replicates using specific quantitative PCR assays. Results were correlated to daily methane production (mL/g VS added), methane proportion of biogas (% $CH_4/(CH_4 + CO_2)$), and acetate concentration using Spearman's Rank (ρ) analysis using R.

	Nearest neighbor (%ID)	vs. Daily methane ρ (p-value)	vs.% Methane ρ (p-value)	vs. [Acetate] ρ (p-value)
OTU2847	*Clostridium leptum* (74)	−0.248 (0.141)	−0.261 (0.197)	0.524 (0.002)
OTU2811	*Clostridium thermocellum* (85)	0.551 (0.001)	0.504 (0.003)	−0.299 (0.076)
OTU2686	*Clostridium thermocellum* (75)	0.599 (0.000)	0.561 (0.000)	−0.427 (0.011)
OTU2941	*Heliobacterium modesticaldum* (74)	0.490 (0.004)	0.547 (0.001)	−0.236 (0.161)
OTU2898	*Acetivibrio cellulolyticus* (78)	0.556 (0.001)	0.550 (0.001)	−0.608 (0.000)
OTU795	*Methanosarcina barkeri* (88)	0.316 (0.061)	0.242 (0.150)	−0.453 (0.007)
OTU805	*Methanoculleus bourgensis* (90)	0.749 (0.000)	0.812 (0.000)	−0.257 (0.129)
Total bacteria		0.340 (0.043)	0.350 (0.038)	−0.066 (0.689)
Total archaea		0.627 (0.000)	0.698 (0.000)	−0.473 (0.005)

10× after the first week, and then declined to its initial level by day 48. While universally abundant, this OTU did not correlate significantly to methane production, but did have a significant positive correlation to acetate accumulation, and is likely involved in the hydrolytic or acidogenic stages of methanogenesis. Two other *Clostridium*-like OTU, 2811 and 2686 (85% and 75% identity to *Clostridium thermocellum* respectively), may have played a more significant role in maximizing digester performance. OTU2686 was present at $\sim 10^5$ genomes/mL digestate at the onset of the trial and continued to increase 100–1000× in the methane-producing

INC, MAN and TST reactors while remaining level in the poorly performing TSM reactors. Both showed a strong, positive correlation to daily methane production as well as the proportion of methane in the biogas samples. In addition, OTU2686 correlated negatively to acetate levels. While OTU2686 was present in the TSM reactors, the lack of proliferation of this organism compared to INC, MAN and TST reactors may be indicative of reduced metabolic activity, contributing to the poor performance. This data suggests a critical role for these *Clostridium*-like organisms in the hydrogenotrophic methanogenesis pathway in this system, potentially as acetate oxidizing syntrophic bacteria, with OTU2811 in particular conspicuously absent in poorly performing digesters.

OTU2898, most closely related to A*cetivibrio cellulolyticus* (78% identity), was not detectable at the onset of the trial, but increased to $\sim10^4$ genomes/mL digestate by day 7, and continued to increase to $\sim10^8$ genomes/mL digestate by day 48 (Fig. 6). This OTU showed a significant positive correlation to methane production in this system as well as a strong negative correlation to acetate accumulation, and was also present at much lower levels in the TSM reactors, appearing only at the last time-point in the trial. The qPCR data for this OTU indicates that this organism was more abundant in the INC and TST libraries than the pyrosequencing data would suggest. It is possible that these sequences are under-represented during universal target amplification for pyrosequencing, or that the OTU-specific primers are co-amplifying an additional unknown sequence, despite the precautions taken to design and validate this OTU-specific assay.

OTU2941, most closely related to *Heliobacterium modesticaldum* (74% identity), was present at very low levels at the onset of the trial ($\sim10^2$ genomes/mL digestate), and increased $\sim$10,000$\times$ in the TST reactors compared to only $\sim$100$\times$ in the INC, MAN and TSM reactors (Fig. 6). While this OTU did have a statistically significant correlation to daily methane production, it was not as strongly positive as those for OTU2686 and OTU2811 (Table 1). However, its proliferation in the TST reactors, particularly in light of the increased performance in comparison to the TSM reactors, suggests a role for this organism in the successful digestion of thin stillage.

3.8. Archaeal OTU (type II chaperonin)

In contrast to total bacterial load, total archaeal load as measured by qPCR correlated both positively to daily methane production and negatively to acetate accumulation (Table 1). OTU805, corresponding to the dominant hydrogenotrophic methanogen in this system (90% identity to *M. bourgensis*) was universally present at 10^7–10^9 genomes/mL digestate, and increased at least 10$\times$ in INC, MAN and TST reactors during the trial while decreasing 10$\times$ in the TSM reactors (Fig. 6). The abundance of this OTU had a strong, significant positive correlation to both daily methane production and the proportion of methane in the biogas, but did not correlate to acetate levels in the digestate, suggesting a metabolic role in hydrogenotrophic methanogenesis.

In contrast, OTU795 was only abundant in the most highly productive reactors, INC and MAN, was present at very low levels in the TSM reactors, and not at all in the TST reactors. This OTU did not correlate to daily methane production, but did correlate negatively to acetate accumulation. This suggests that OTU795 (88% identity to *M. barkeri*) represents an acetoclastic methanogen, and its proliferation in the INC and MAN reactors likely explains the complete consumption of acetate in those digesters. However, the lack of correlation of OTU795 abundance to daily methane production does suggest that the hydrogenotrophic pathway was the dominant methanogenic pathway in this system.

Given that the archaea required for methane production were present based on qPCR data (Fig. 6F and G), it is unclear whether the lack of methanogenesis in the TSM digesters was due to (i) outgrowth of inhibitory microorganisms (ii) absence of microorganisms fulfilling compulsory metabolic functions or (iii) an inhibitory substrate in the reactors, for example ammonia, suppressing archaeal growth and function (Westerholm et al., 2011). There is also the possibility that while the DNA was detectable, the archaea in the non-functioning digesters were not viable.

It is likely that at pH < 6.5 and in the absence of a significant population of acetoclastic methanogens, the most likely mode of methane production in both the TST and TSM reactors was via the syntrophic acetate oxidation pathway, with the resulting H_2 and CO_2 converted to methane by the large population of hydrogenotrophic methanogens (Delbès et al., 2001). Given the persistent acetate accumulation in the TSM digesters, it is possible that the absence of bacteria able to fulfill this metabolic niche under these reactor conditions resulted in poor archaeal methanogenic activity and growth. Quantitative PCR identified two OTU, OTU2686 and OTU2898, both of which correlated negatively to acetate accumulation and positively to daily methane production that could possibly be fulfilling this role. Syntrophic acetate oxidation is not energetically favorable, and is only feasible when coupled with hydrogenotrophic methanogenesis; when H_2 concentrations are high, this reaction is reversed, with bacteria creating acetate from H_2 and CO_2 (Hattori, 2008). The fluctuation between these two methanogenesis pathways is often dependent on environmental and operating conditions. Hydrogenotrophic methanogens tend to be more abundant in thermophilic, low pH, high acetate environments, are more resistant to ammonia accumulation in the digestate, and are often the most abundant methanogens when these conditions are present in the reactor (Hattori, 2008). The lack of acetoclastic methanogens in the TST and TSM reactors and consequent reliance on hydrogenotrophic methanogenesis for methane production may explain their reduced performance compared to INC and MAN reactors, which had significant populations of acetoclastic methanogens able to convert acetate to methane directly, a much more energetically favorable reaction. The reversibility of the hydrogenotrophic reaction may also explain the concurrent methane and acetate accumulation in the TST reactors near the end of the trial period.

Further insight into the conditions that are favorable for proliferation of both hydrogenotrophic and acetoclastic methanogens may provide a methanogenic community that is more robust and productive.

4. Conclusion

Large-scale shifts were observed in the bacterial community while the archaeal community remained stable. During digestion, the microbial communities shifted towards a common phylogenetic structure, regardless of digester input. Differences in OTU frequency between methane-producing and non-methane-producing reactors provided insight into the bacterial species necessary for acetate catabolization and a successful transition from acidogenesis to methanogenesis. Reactors processing manure contained significant populations of acetoclastic and hydrogenotrophic methanogens, while only hydrogenotrophic methanogens were detected in reactors processing stillage. Quantitative PCR also confirmed the relationship of specific OTU to reactor performance measures including VFA production and catabolization and methane production.

Acknowledgements

We gratefully acknowledge the support of John Schellenberg and Richard Sparling for the VFA analysis. This work was supported

by the ecoENERGY Innovation Initiative (Natural Resources Canada).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2013.10.070.

References

Agler, M.T., Garcia, M.L., Lee, E.S., Schlicher, M., Angenent, L.T., 2008. Thermophilic anaerobic digestion to increase the net energy balance of corn grain ethanol. Environ. Sci. Technol. 42 (17), 6723–6729.

American Public Health Association, 1995. Standard Methods for the Examination of Water and Wastewater, 20th ed..

Chaban, B., Hill, J.E., 2012. A 'universal' type II chaperonin PCR detection system for the investigation of Archaea in complex microbial communities. ISME J. 6 (2), 430–439.

Dar, S.A., Kleerebezem, R., Stams, A.J.M., Kuenen, J.G., Muyzer, G., 2008. Competition and coexistence of sulfate-reducing bacteria, acetogens and methanogens in a lab-scale anaerobic bioreactor as affected by changing substrate to sulfate ratio. Appl. Microbiol. Biotechnol. 78 (6), 1045–1055.

Delbès, C., Moletta, R., Godon, J.-J., 2001. Bacterial and archaeal 16S rDNA and 16S rRNA dynamics during an acetate crisis in an anaerobic digestor ecosystem. FEMS Microbiol. Ecol. 35 (1), 19–26.

Demirel, B., Scherer, P., 2008. The roles of acetotrophic and hydrogenotrophic methanogens during anaerobic conversion of biomass to methane: a review. Rev. Environ. Sci. Biotechnol. 7 (2), 173–190.

Dumonceaux, T.J., Hill, J.E., Hemmingsen, S.M., Van Kessel, A.G., 2006. Characterization of intestinal microbiota and response to dietary virginiamycin supplementation in the broiler chicken. Appl. Environ. Microbiol. 72 (4), 2815–2823.

Eskicioglu, C., Kennedy, K.J., Marin, J., Strehler, B., 2011. Anaerobic digestion of whole stillage from dry-grind corn ethanol plant under mesophilic and thermophilic conditions. Bioresour. Technol. 102 (2), 1079–1086.

Gannoun, H., Khelifi, E., Omri, I., Jabari, L., Fardeau, M.L., Bouallagui, H., Godon, J.J., Hamdi, M., 2013. Microbial monitoring by molecular tools of an upflow anaerobic filter treating abattoir wastewaters. Bioresour. Technol. 142, 269–277.

Hattori, S., 2008. Syntrophic acetate-oxidizing microbes in methanogenic environments. Microbes Environ. 23 (2), 118–127.

Hill, J.E., Penny, S.L., Crowell, K.G., Goh, S.H., Hemmingsen, S.M., 2004. CpnDB: a chaperonin sequence database. Genome Res. 14 (8), 1669–1675.

Hill, J.E., Town, J.R., Hemmingsen, S.M., 2005. Improved template representation in *cpn60* polymerase chain reaction (PCR) product libraries generated from complex templates by application of a specific mixture of PCR primers. Environ. Microbiol. 8 (4), 741–746.

Johnson, E.A., Madia, A., Demain, A.L., 1981. Chemically defined minimal medium for growth of the anaerobic cellulolytic thermophile *Clostridium thermocellum*. Appl. Environ. Microbiol. 41 (4), 1060–1062.

Krause, L., Diaz, N.N., Edwards, R.A., Gartemann, K.H., Kromeke, H., Neuweger, H., Puhler, A., Runte, K.J., Schluter, A., Stoye, J., Szczepanowski, R., Tauch, A., Goesmann, A., 2008. Taxonomic composition and gene content of a methane-producing microbial community isolated from a biogas reactor. J. Biotechnol. 136 (1–2), 91–101.

Lee, D.H., Zo, Y.G., Kim, S.J., 1996. Nonradioactive method to study genetic profiles of natural bacterial communities by PCR-single-strand-conformation polymorphism. Appl. Environ. Microbiol. 62 (9), 3112–3120.

Levin, D.B., Islam, R., Cicek, N., Sparling, R., 2006. Hydrogen production by *Clostridium thermocellum* 27405 from cellulosic biomass substrates. Int. J. Hydrogen Energy 31 (11), 1496–1503.

Links, M., Chaban, B., Hemmingsen, S., Muirhead, K., Hill, J., 2013. MPUMA: a computational approach to microbiota analysis by de novo assembly of operational taxonomic units based on protein-coding barcode sequences. Microbiome 1 (1), 23.

Links, M.G., Dumonceaux, T.J., Hemmingsen, S.M., Hill, J.E., 2012. The chaperonin-60 universal target is a barcode for bacteria that enables *de novo* assembly of metagenomic sequence data. PLoS One 7 (11), e49755.

Liu, F.H., Wang, S.B., Zhang, J.S., Zhang, J., Yan, X., Zhou, H.K., Zhao, G.P., Zhou, Z.H., 2009. The structure of the bacterial and archaeal community in a biogas digester as revealed by denaturing gradient gel electrophoresis and 16S rDNA sequencing analysis. J. Appl. Microbiol. 106 (3), 952–966.

Luo, G., Xie, L., Zhou, Q., Angelidaki, I., 2011. Enhancement of bioenergy production from organic wastes by two-stage anaerobic hydrogen and methane production process. Bioresour. Technol. 102 (18), 8700–8706.

Lv, W., Zhang, W., Yu, Z., 2013. Evaluation of system performance and microbial communities of a temperature-phased anaerobic digestion system treating dairy manure: thermophilic digester operated at acidic pH. Bioresour. Technol. 142, 625–632.

Madden, R.H., 1983. Isolation and characterization of *Clostridium stercorarium* sp. nov., cellulolytic thermophile. Int. J. Syst. Bacteriol. 33 (4), 837–840.

Mustafa, A.F., McKinnon, J.J., Ingledew, M.W., Christensen, D.A., 2000. The nutritive value for ruminants of thin stillage and distillers' grains derived from wheat, rye, triticale and barley. J. Sci. Food Agric. 80 (5), 607–613.

Schaefer, S.H., Sung, S., 2008. Retooling the ethanol industry: thermophilic anaerobic digestion of thin stillage for methane production and pollution prevention. Water Environ. Res. 80 (2), 101–108.

Schauer-Gimenez, A.E., Zitomer, D.H., Maki, J.S., Struble, C.A., 2010. Bioaugmentation for improved recovery of anaerobic digesters after toxicant exposure. Water Res. 44 (12), 3555–3564.

Sleat, R., Mah, R.A., Robinson, R., 1984. Isolation and characterization of an anaerobic, cellulolytic bacterium, *Clostridium cellulovorans* sp. nov. Appl. Environ. Microbiol. 48 (1), 88–93.

Wang, H., Tolvanen, K., Lehtomäki, A., Puhakka, J., Rintala, J., 2009. Microbial community structure in anaerobic co-digestion of grass silage and cow manure in a laboratory continuously stirred tank reactor. Biodegradation 21 (1), 135–146.

Ward, A.J., Hobbs, P.J., Holliman, P.J., Jones, D.L., 2008. Optimisation of the anaerobic digestion of agricultural resources. Bioresour. Technol. 99 (17), 7928–7940.

Weiland, P., 2010. Biogas production: current state and perspectives. Appl. Microbiol. Biotechnol. 85 (4), 849–860.

Werner, J.J., Knights, D., Garcia, M.L., Scalfone, N.B., Smith, S., Yarasheski, K., Cummings, T.A., Beers, A.R., Knight, R., Angenent, L.T., 2011. Bacterial community structures are unique and resilient in full-scale bioenergy systems. Proc. Natl. Acad. Sci. USA 108 (10), 4158–4163.

Westerholm, M., 2012. Improved biogas production from whole stillage by co-digestion with cattle manure. Bioresour. Technol..

Westerholm, M., Dolfing, J., Sherry, A., Gray, N.D., Head, I.M., Schnürer, A., 2011. Quantification of syntrophic acetate-oxidizing microbial communities in biogas processes. Environ. Microbiol. Rep. 3 (4), 500–505.

Ziganshin, A., Liebetrau, J., Pröter, J., Kleinsteuber, S., 2013. Microbial community structure and dynamics during anaerobic digestion of various agricultural waste materials. Appl. Microbiol. Biotechnol. 97 (11), 5161–5174.

Ziganshin, A., Schmidt, T., Scholwin, F., Il'inskaya, O., Harms, H., Kleinsteuber, S., 2011. Bacteria and archaea involved in anaerobic digestion of distillers grains with solubles. Appl. Microbiol. Biotechnol. 89 (6), 2039–2052.