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Performance of thermophilic anaerobic digesters using inoculum mixes with enhanced methanogenic diversity

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Abstract

BACKGROUND: Various mixes of seeds have been quasi-randomly selected to startup anaerobic digesters. In contrast, this study examines the impact of inoculating thermophilic anaerobic digesters with a designed mix of non-acclimated seeds based on their methanogen composition, using quantitative polymerase chain reaction (QPCR) of 16S rRNA gene, to achieve high abundance and diversity of methanogens.

RESULTS: Based on QPCR results, two seed mixes were selected to inoculate two anaerobic digesters: digester (A) was inoculated with a control seed consisting of digestate, manure, and activated sludge; and digester (B) was inoculated with a further methanogen-enriched seed consisting of the control seed with added compost and leachate. Both seed combinations yielded a balanced microflora that is able to achieve a successful startup. However, upon reaching steady state, digester B exhibited lower propionate levels, resulting in lower volatile fatty acids (VFAs) concentration and increased buffering capacity, indicating greater stability. Acetotrophs and hydrogenotrophs were dominated by *Methanosarcinaceae* and *Methanobacteriales*, respectively, in both digesters, exhibiting an average ratio of 66-to-34% in A and 76-to-24% in B during steady-state.

CONCLUSION: The inoculation strategy in digester B resulted in improved stability, lower propionate concentration and 10% higher relative abundance of acetotrophs.
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Keywords: anaerobic digestion; biomass; waste treatment and minimization

INTRODUCTION

Anaerobic digestion (AD) systems operating under thermophilic conditions (55–60 °C) have the unique feature of producing hygienic fertilizers with greater process efficiency. However, to date, the market penetration of thermophilic AD has been hindered by operational difficulties and instability challenges invariably associated with a poor startup and lack of acclimated inoculum. As such, readily available seeds have been suggested to enhance the startup of anaerobic digesters including, but not limited to, primary and secondary sludge, waste activated sludge (WAS), wastewater, landfill leachate, municipal compost, rumen and raw manure.[1]

Several studies examined the importance of the appropriate source and type of seed. [2–4] The optimum inoculum depends greatly on the substrate composition and operating conditions. Cordoba *et al.* [1] reported that stabilized swine wastewater and sewage sludge are more effective inoculation sources than rumen, as they can lead to improved solids removal and methane generation in mesophilic AD of swine wastewater. Forster-Carneiro *et al.*[4] tested six inoculum sources (corn silage, digested waste with rice hulls, cattle excrement, swine excrement, and a mix of swine excrement and sludge) and concluded that the digested sludge from a mesophilic anaerobic digester, was the best inoculum source for thermophilic AD of the organic fraction of municipal solid waste (OFMSW). In addition, Costa *et al.* [2] showed that the operational mode of the seeding system (i.e. suspended vs granular) can also affect the inoculated digester. They showed that the optimum inoculum mix for mesophilic AD of *Ulexeuropaeus*, a widely invasive plant, consists of 55% anaerobic granular sludge to 45% anaerobic suspended sludge. Furthermore, Moset *et al.*[3] showed that the substrate on which the seeding system feeds can also have a significant impact. Anaerobic systems fed with more than 70% animal manure provided optimum inocula that can result in the highest methane production, for thermophilic AD of wheat straw, whole crop maize, cattle manure, grass and cellulose. Similarly, Gu *et al.*[5] showed that seeds from manure digesters are better inocula for the mesophilic AD of rice straw, compared with seeds from sludge digesters. Also, digesters treating food leftovers can provide a better seed, leading to a higher methane yield, compared with seeds from digesters treating wastewater sludge.[6]

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Other studies considered combining more than one source/type of seed to reach an optimum mix with a diverse microbial composition. In this context, using manure alone to inoculate thermophilic AD of the OFMSW resulted in high propionate accumulation[7] while enriching cattle manure with soil and WAS resulted in a noticeably faster startup of thermophilic digesters than those seeded with manure only.[8] Alternatively, several types of manure, e.g. horse, dog and sheep feces, were mixed to enhance the anaerobic 'co-metabolism' of fruit and vegetable waste.[9]

Other sources, less studied than animal manure, that have the potential to enrich the initial seed include municipal waste compost and landfill leachate. In fact, municipal waste compost can host thermophilic methanogens in anoxic niches, at a level of $\sim 2 \times 10^7$ to 10^9 g^{-1} dry weight. Methanogens in 6-week-old samples of biowaste compost at 60 °C were associated with the hydrogenotrophic methanogen *Methanothermobacter spp*. (86%) and the acetoclastic methanogen *Methanosarcina thermophila spp*. (14%).[10,11] The addition of the waste compost to a mesophilic digester, inoculated with manure and sewage sludge, induced a shift in the methanogenic community toward hydrogenotrophs and reduced the required hydraulic retention time.[12]

Similarly, it has been shown that methanogens constitute 2% of the total microbial community in leachate from a solid waste landfill and that dominant clones in landfills are close relatives to thermophilic hydrogenotrophic methanogens.[13,14] In addition, leachate can be used for its ability to enhance cellulose hydrolysis.[15] Yet, leachate is reported to be a less efficient inoculum, compared to anaerobic sludge for mesophilic AD of solid agro-industrial waste.[16]

While several studies aimed at providing a random mix of non-acclimated seeds to optimize the performance of thermophilic anaerobic digesters, this study attempts for the first time to rationally design a mix of non-acclimated seeds based on their methanogen composition, using quantitative polymerase chain reaction (QPCR) of 16S rRNA gene, to achieve high abundance and diversity of methanogens. To the best of our knowledge, the impact of adding concomitantly municipal compost and landfill leachate on the startup and steady-state operation of thermophilic AD of the OFMSW has not been reported previously. The digesters were operated in a semi-continuous feeding mode for 250 days to evaluate the seed impacts on the performance, stability, and methanogenic composition during startup and steady-state operation.

MATERIALS AND METHODS

Waste collection and characterization

The OFMSW was collected from local households and food markets then ground and homogenized using a commercial food processor. The waste was prepared in one large batch, stored at -20 °C in small containers, and used for feeding throughout the experimental program to minimize fluctuations in the feed characteristics. Three samples were collected from the waste batch and analyzed for particulate and soluble compounds prior to storage. Average values are reported in Table 1. The required amount of waste was thawed before feeding to avoid aerobic degradation, which might alter the feedstock composition.

Table 1. Feed characteristics

Parameter	Value	Unit
Particulates[a]		
TS	29.3	%
VS	95.5	% TS
COD_{Total}	467.5	g/L
Carbon[b]	41.8	%
Total Organic Carbon[b]	37.8	%
Nitrogen[b]	2.7	%
C:N	15	
Total Phosphorus	1.7	g kg^{-1}
Soluble compounds[c]		
$COD_{Soluble}$	14.0	g L^{-1}
NO_3_N	13.4	mg L^{-1}
NO_2_N	0.05	mg L^{-1}
NH_3	19.0	mg L^{-1}
Partial Alkalinity, pH = 5.75	0.0	mg L^{-1}
Total Alkalinity, pH = 4.3	432.0	mg L^{-1}
Acetate	170.5	mg L^{-1}
Propionate	648.0	mg L^{-1}
Butyrate	37.5	mg L^{-1}

[a] Concentrations in the raw feed, i.e. prior to dilution with distilled water for feeding.
[b] Values determined on dry basis.
[c] Concentrations in centrifuged and filtered supernatant upon dilution of 107 g of raw OFMSW with distilled water to a volume of 700 mL to achieve OLR = 2 g VS L^{-1} d^{-1} and HRT = 30 days.

System set up and operation

Two anaerobic reactors (Bioflo 110, New Brunswick Scientific Co.), of 14 L capacity and 9 L working volume, were operated at 55 °C and were continuously mixed with an internal impeller at 80 rpm – a stirring speed that was proven adequate for the stable operation of thermophilic AD of OFMSW.[7,17] The organic loading rate (OLR) was step-increased by 20–35% every three weeks, from 0.8 to 2.4 gVS L^{-1} d^{-1}, with no observed accumulation of volatile fatty acids (VFAs). The hydraulic retention time (HRT) was maintained at ~45 days till day 51 of the experimental programme and was then reduced to 30 days and kept constant afterwards. A volume of 700 mL was wasted from the digesters, three times per week, to achieve the average HRT of 30 days. The digesters were batch fed with an equal volume of diluted waste, consisting of de-ionized water mixed with the appropriate weight of OFMSW, to achieve the target OLR of 2.4 gVS L^{-1} d^{-1}. The adopted HRT and design OLR, as well as the incremental feeding strategy were defined based on previous successful startup of thermophilic AD of OFMSW in the absence of acclimated inocula.[7] After every feeding, the digesters' headspace was purged with nitrogen for 2 min to avoid oxygen contamination.

Inoculation process

Digester A was inoculated with a control seed mix consisting of anaerobic digestate (from a thermophilic lab reactor inoculated with manure and fed with OFMSW), cattle manure (from a local cattle rearing farm a few hours after excretion), and WAS (from a nearby wastewater treatment plant) to provide a diverse consortium of methanogens. Digester B was inoculated with the control mix supplemented with landfill leachate (from an active solid waste landfill) and municipal waste compost (from a nearby active composting plant with an unfinished windrow reaching ~60 °C) to further improve the biodiversity of the inoculum.

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Table 2. QPCR primers and probes used to quantify various methanogenic archaea

Primer-probe	Target	Plasmid standard	Sequence 5′-3′
MBT 857 F	*Methanobacteriales*	*Methanobacteriumformicium* (EU 544028)	CGWAGGGAAG CTGTTAAGT
MBT 1196 R			TACCGTCGTCCACTCCTT
MBT 929 probe			AGCACCACAACGCGTGGA
MMB 282 F	*Methanomicrobiales*	*Methanomicrobiales KB 1-1* (AN – DQ301905)	ATCGRTACGGGTTGTGGG
MMB 832 R			CACCTAACGCRCATHGTTTAC
MMB 749 probe			TYCGACAGTGAGGRACGAAAGCTG
MST 702 F	*Methanosaetaceae*	*Methanosaeta KB 1-1* (AY 570685)	TAATCCTYGARGGACCACCA
MST 863 R			CCTACGGCACCRACMAC
MST 753 probe			ACGGCAAGGGACGAAAGCTAGG
MSC 380 F	*Methanosarcinaceae*	*Methanosarcinathermophila* (M59140)	GAAACCGYGATAAGGGGA
MSC 828R			TAGCGARCATCGTTTACG
MSC 492 probe			TTAGCAAGGGCCGGGCAA

The collection and addition of seeds to the digesters took 24 days. On the first day, 1250 g of raw manure were introduced into the empty reactors and diluted with 4.5 L of wasted digestate. On the third day, 1 L of WAS was added to each digester. Both digesters were then filled with de-ionized water to reach a working capacity of 9 L. On day 9, 1.3 L of digester B was replaced with leachate, while 1.3 L of digester A was replaced with de-ionized water. On day 24, 100 g of compost were added to digester B. The temperature in the reactors was increased to 55 °C on day 1, without a gradual acclimation period, thus selecting real thermophiles rather than thermotolerant microogansims.[20] The 'seeding strategy', as referred to in the text, represents all aspects related to seeding (i.e. seed source, seed mix and frequency of seeding).

Monitoring and control methods

The temperature control was automated through a control unit connected to a built-in temperature probe and a heating blanket. Continuous pH reading was conducted using a submerged pH probe with manual adjustment (when needed) by adding NaOH (5 mol L^{-1}) solution. Biogas measurements were taken once or twice daily, using the water displacement method for total gas generation and a dual wavelength infrared cell with reference channels (GEM-2000 monitor, Keison Products, UK) for CH_4 concentration. The latter is multiplied by the total gas generation to obtain the CH_4 generation rate. The specific methane yield was calculated as liters of methane generated per gram of VS fed into the system.

The biochemical analysis was conducted on a weekly basis whereby a fraction of the wasted material was used to measure total and volatile solids, and total chemical oxygen demand (COD). The rest was centrifuged and filtered using a 1.2 µm pores filter for analysis of soluble compounds. COD (total and soluble) was determined using the modified 5220 D procedure of standard methods for the analysis of water and wastewater (APHA, AWWA and WPCF, 2012) by HACH high-range COD kit (HACH Company, Loveland, Colorado). Similarly, solid content (total, suspended, dissolved and volatile) was analyzed using Standard Methods 2540B and 2540E procedures. Partial and total alkalinities (PA and TA, respectively) were measured by titration with HCl (0.2 N) to pH of 5.75 and 4.3, respectively, and intermediate alkalinity (IA) was calculated as the difference between PA and TA.[21] Total ammonia content was determined by spectrophotometry using HACH high-range ammonia kit (HACH Company, Loveland, Colorado). The free ammonia (NH_3) was calculated based on the NH_3-to-NH_4^+ relationships expressed in Equations (1) and (2):[22]

$$NH_3 - N = \frac{NH_4^+ - N \times 10^{pH}}{K_b/K_w + 10^{pH}} K_b/K_w \quad (1)$$

$$NH_3 - N = e^{(6344/273+T)} \quad (2)$$

where K_b/K_w is the ratio of the ionization constant of the ammonia equilibrium equation to the ionization constant of water.

The concentrations of acetate, propionate and butyrate inside the digesters were measured, once to twice per HRT, using a gas chromatograph (Trace GC Ultra, Thermo Electron Corporation) equipped with a flame ionization detector and a TR-FFAP capillary column with nitrogen carrier gas. The temperature inside the oven was initially set at 85 °C (3 min hold time) and increased at a rate of 6 °C min^{-1} to a final temperature of 150 °C (1 min hold time). Inlet and FID base temperatures were set to 240 °C and 220 °C, respectively.

Quantitative polymerase chain reaction (QPCR)

Total genomic DNA was extracted from the samples using the Power soil DNA isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA) according to the manufacturer's instructions. The proportions of various methanogens, *Methanobacteriales*, *Methanomicrobiales*, *Methanosaetaceae* and *Methanosarcinaceae*, in the total methanogenic community, were determined by QPCR. Amplification was performed in a 25 μL reaction volume containing 12.5 μL of 2 × iQ Supermix, 0.5 µmol L^{-1} of each primer, 0.2 µmol L^{-1} of each probe, 1 μL sample DNA, and RNase-free sterile water to a final volume of 25 μL. Amplification was performed using the CFX96 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA) with PCR conditions for methanogens described by Yu *et al.* [23] All primers, probes and plasmid standards used in this study are listed in Table 2. For standard clone preparation, the PCR amplicons were first cloned into a TOPO cloning vector (pCR 2.1-Topo vector, Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Plasmids from transformed cells were extracted by the PureYield™ Plasmid Miniprep System (Promega, Madison, WI). Copy numbers per microliter were calculated from the concentration of extracted plasmid DNA using the known sequences of the vector and inserts. The standard template DNA was diluted in series and the C_t values for each dilution were plotted against

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Table 3. Relative abundance of various methanogenic orders in the selected seeds

Seed	Relative abundance[a] (%)					
	MBT[b]	MMB[b]	MSC[b]	MST[b]	Hydrogenotrophs	Acetotrophs
Digestate	5	-[c]	95	<1	5	95
Manure	15	-	85	-	15	85
WAS	3	50	2	46	53	48
Compost	22	-	41	37	22	78
Leachate	<1	94	6	<1	94	6

[a] Percentage of total number of methanogenic 16S rRNA gene copies/ng DNA.
[b] MBT *Methanobacteriales*; MMB: *Methanomicrobiales*; MSC: *Methanosarcinaceae*; MST: *Methanosaetaceae*.
[c] Below the detection limit of the qPCR assays.

the 16S rRNA gene copy number of each dilution to construct the standard curve. All QPCR assays were performed in triplicate. QPCR assays with C_t values over 40 were considered negative. For each QPCR run, a negative (no template) control was used to test for false positives or contamination.

RESULTS AND DISCUSSION

Seed selection and characterization

Both acetotrophic methanogens (converting acetate to CH_4) and hydrogenotrophic methanogens (converting H_2/CO_2 into CH_4) are essential for AD systems. While acetotrophs often dominate, hydrogenotrophic methanogens are known to be more tolerant to acid accumulation and favored under thermophilic conditions.[18] Acetotrophic methanogenesis is achieved by members of the family *Methanosarcinaceae* (with higher growth rate) at high acetate concentrations, or *Methanosaetaceae* (with higher substrate affinity) at low acetate concentrations (<1 mmol).[19] Therefore, initial seeds were selected to provide both acetotrophic families. Similarly, inocula containing both hydrogenotrophic orders (*Methanobacteriales* and *Methanomicrobiales*) were included in the seeding mixes. In addition to enriching the methanogenic component, combining several seeding sources increases the chances of an overall diverse microbial community that offers the advantage of better and faster degradation of organic compounds due to syntrophic interactions where the waste of one species is needed by other species.[9]

Five types of seeding sources were tested using QPCR: thermophilic digestate, cattle manure, WAS, compost and leachate. The digestate and manure exhibited a very close methanogenic composition. They were both dominated by the *Methanosarcinaceae* family (95% and 85%, respectively), of the acetotrophic *Methanosarcinales* order, with a limited presence of the hydrogenotrophic *Methanobacteriales* order (5% and 15%, respectively) (Table 3). The observed similarity may be due to drawing the digestate from a digester that was inoculated with manure only. The control seed mix consisted of manure, digestate, and WAS. The latter provided a source of *Methanomicrobiales* (50%) and *Methanosaetaceae* (46%), both absent in the digestate and manure.

The compost (78% acetotrophs) and leachate (94% hydrogenotrophs, mainly Methanomicrobiales) were added to the control seed mix components with the aim to improve the seed mix by enhancing the methanogenic diversity of the inoculum through an additional source of hydrogenotrophic orders and the less abundant acetotrophic family (Methanosaetaceae).

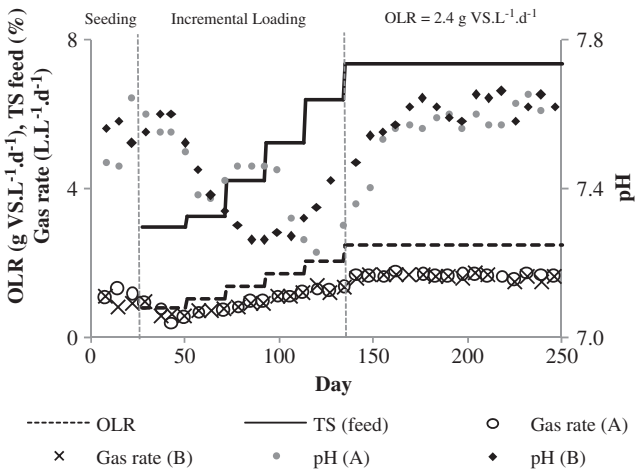

Figure 1. Loading conditions, pH, and weekly average gas generation.

While similar hydrogenotrophic dominance in landfill leachate has been reported,[13,14] the compost used in this experiment was dominated by acetotrophs, in contrast to previously reported hydrogenotrophic dominance in domestic waste compost.[11]

Startup

During the seeding period, feeding was varied depending on the performance of the digesters (attempting to maintain methane generation while avoiding VFA buildup), resulting in average biogas generation rates of 1.15 L L^{-1} d^{-1} and 0.99 L L^{-1} d^{-1} in digesters A and B, respectively. At the end of the seeding period (day 26), incremental loading was initiated by gradually increasing the weight of the waste in the feed, while keeping a constant influent and effluent volume to maintain a constant HRT. Accordingly, the OLR was increased gradually to reach a design rate of 2.4 gVS L^{-1} d^{-1} (Fig. 1). Upon initiation of incremental loading, the weekly average biogas volume dropped because of the low initial OLR (0.8 gVS L^{-1} d^{-1}) and increased again with organic loading to stabilize at ~1.66 L L^{-1} d^{-1} in A and 1.60 L L^{-1} d^{-1} in B at OLR = 2.4 gVS L^{-1} d^{-1} (Fig. 1).

On the other hand, pH increased during the seeding period, presumably because of enhanced acid degradation with the progressive addition of seeds. During the incremental loading period, the pH decreased to ~7.2 then gradually increased, indicating the progressive acclimation of the microbial flora, until stabilizing at ~7.6 in both digesters. Note that the rising slope of the pH graph in digester B started about 30 days before digester A; and upon

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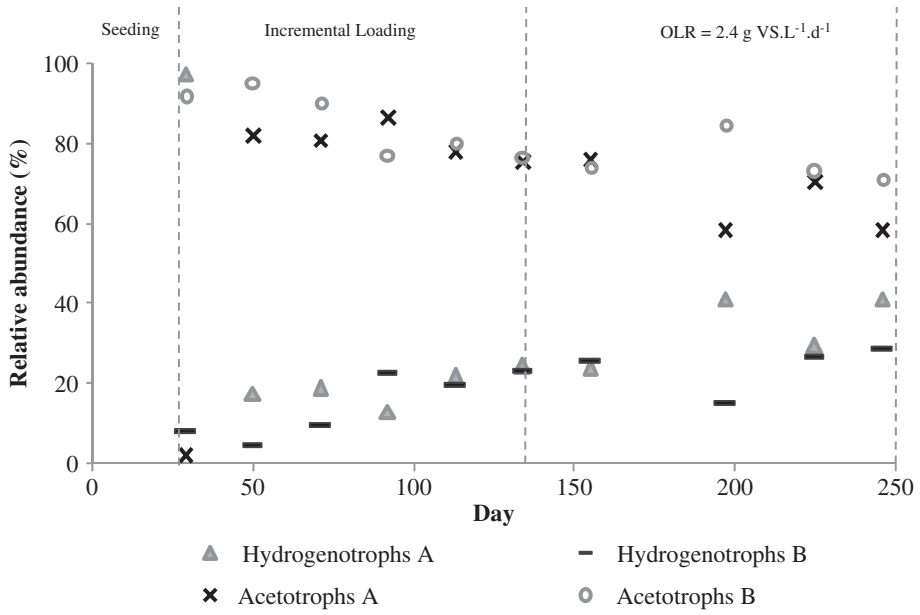

Figure 2. Relative abundance of hydrogentrophs and acetotrophs. *(percentage of total number of methanogenic gene copies per ng extracted DNA)*

reaching steady state, the pH in B was always slightly higher than that in digester A (Fig. 1) which can be attributed to faster microbial acclimation in digester B during startup and lower acid generation during steady-state. The target OLR (2.4 gVS L^{-1} d^{-1}) was constantly applied over the last 115 days (~4 HRT) during which the pH was stable in both digesters and the variation between two consecutive gas readings was <10% (0.1 to 6.9% in A and 0.2 to 9.8% in B), indicating a steady-state operation.[24,25]

In general, both digesters were successfully started in 3.6 months, from day 26 (time needed to acquire all seeds) to 135 (initiation of stable feeding), with final OLR = 2.4 gVS L^{-1} d^{-1} and HRT = 30 days. In thermophilic digesters with a seed that is not acclimated to high temperatures and to the OFMSW substrate, the startup can take longer.[26] In fact, the start-up of a similar digester, inoculated only with manure, required 5.6 months to reach the target OLR of 2.5 gVS L^{-1} d^{-1}.[7]

Microbial analysis

In both digesters, the methanogenic community was dominated by acetotrophic methanogens (Fig. 2), consisting almost solely (100%) of *Methanosarcinaceae*. The hydrogenotrophic methanogens consisted mainly (95–100%) of *Methanobacteriales*, typical of similar thermophilic anaerobic digesters.[27,28] *Methanomicrobiales* were present at trace levels (<1%) whereas *Methanosaetaceae* were absent, even though both were present in the initial seeds. The addition of WAS (50% *Methanomicrobiales* and 46% *Methanosaetaceae*) and leachate (94% *Methanomicrobiales*) did not contribute to their abundance in the digesters suggesting that the operating conditions and substrate composition are not favorable for the proliferation of these methanogens.

At the onset of the experiment, acetotrophic methanogens had a high relative abundance (>80%) compared to hydrogenotrophic methanogens (<20%). With time, the relative abundance of hydrogenotrophic methanogens increased under acetotrophic dominance. At the end of the experiment (day 250), the difference decreased to reach about 60-to-40% and 70-to-30% acetotrophs-to-hydrogenotrophs, in digesters A and B, respectively (Fig. 2). Accordingly, the addition of leachate and compost seems to have shifted the microbial community by ~10% toward acetotrophs. This observation differs from reported findings in mesophilic systems, where the addition of hydrogenotroph-rich compost resulted, at the end of the experiment, in a shift toward hydrogenotrophic methanogens.[12] However, in this study, the hydrogenotroph-rich leachate, added to digester B, was 94% dominated by the *Methanomicrobiales* order, which did not proliferate, presumably because of selective operational conditions and substrate composition.

COD, solids and ammonia

In the early stages, soluble COD concentrations in digester B were higher than those in digester A because of the additional seeding material. However, during incremental loading (i.e. days 50 to 130) the soluble COD was significantly higher ($P = 0.0003$, student's t-test) in A (3.17 ± 0.95 g L^{-1}) than B (2.03 ± 0.55 g L^{-1}) implying enhanced degradation of soluble compounds in B (Fig. 3(a)). However, the total COD was comparable in both digesters (average =17 g L^{-1}), which could be attributed to the presence of recalcitrant and inorganic material from the compost and/or leachate seed in digester B. During sieving of the compost seed, prior to addition to the digester, small plastic, soil and glass particles were present and removed to the extent visually discernible. The total COD in digester B decreased below that of digester A with progressive washout of the initial seed material (Fig. 3(b)).

Under steady-state operations, the average total COD in digester B was 22 g L^{-1} (of which 3.6 g L^{-1} were soluble), compared with 28 g L^{-1} (4.4 g L^{-1} soluble) in digester A. The COD removal in A and B was 74% and 76%, respectively. The addition of leachate has reportedly improved the hydrolysis of cellulose,[15] which commonly constitutes 9–10% food waste.[29,30] While the slight improvement in COD removal efficiency in digester B (76% vs 74% in A) may be attributed to improved cellulose hydrolysis, the difference is within the margin of error of the measurements and the results can be considered comparable.

Similarly, the total solids (TS) was initially higher in digester B because of additional solids from the compost and leachate

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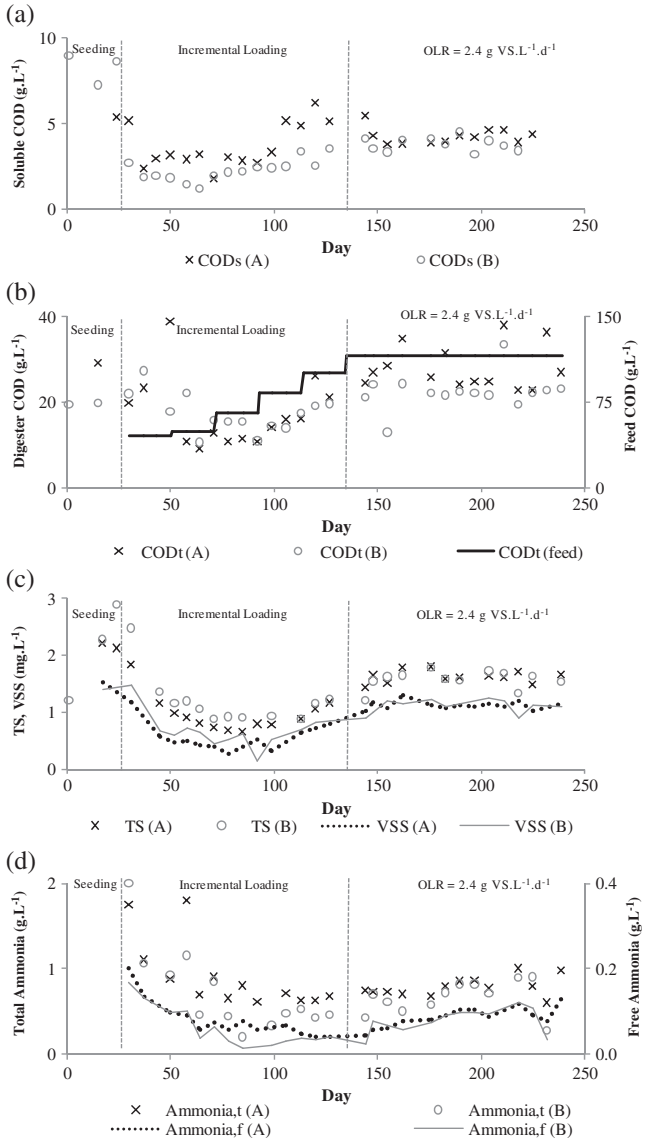

Figure 3. COD, solids, and ammonia concentrations. *(t: total; s: soluble; f: free)*

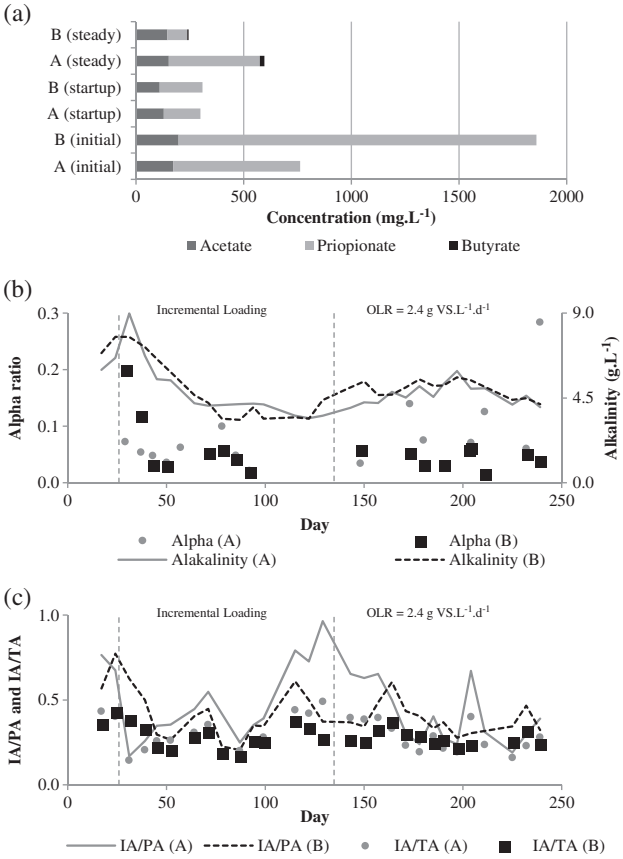

Figure 4. Volatile fatty acids and alkalinity concentrations.

seeds; but eventually converged in both digesters and stabilized at an average of 1.6%, indicating similar TS removal in both digesters during steady state (Fig. 3(c)). Concurrently, volatile suspended solids (VSS), commonly used as an indicator of total microbial abundance in anaerobic reactors,[31] became similar in both digesters with an average of 1.1% at steady state (Fig. 3(c)). However, the pH, which decreased in both digesters upon initiation of incremental loading, increased again at a faster rate, and at an earlier time, in B (Fig. 1), indicating faster microbial acclimation.

Last, while total and free ammonia concentrations in both digesters were below reported inhibitory levels of 1200–4900 mg L^{-1} [20] and 680–690 mg L^{-1},[32] respectively, total ammonia was 23% higher in digester A, with an average of 822 mg L^{-1} (81 mg L^{-1} free ammonia) compared with 667 mg L^{-1} (68 mg L^{-1} free ammonia) in digester B (Fig. 3(d)).

Alkalinity and VFAs

Initially, VFAs were higher in digester B (1545 mg HAc L^{-1} in B vs 651 mg HAc L^{-1} in A), possibly because of the degradation of the additional inocula (compost and leachate). With progressive washout of the initial seeding material, the VFAs decreased and converged in both digesters to 268 and 271 mg HAc L^{-1} in A and B, respectively, almost evenly split between acetate and propionate (Fig. 4(a)). At steady-state, digester B maintained a fairly stable VFA concentration (229 ± 84 mg L^{-1}) composed of acetate (147 ± 50 mg L^{-1}) and propionate (92 ± 63 mg L^{-1}). In contrast, the VFA level was variable in digester A (510 ± 205 mg L^{-1}), and mostly dominated by propionate (423 ± 266 mg L^{-1}). Based on t-test, the propionate concentrations in digester A were significantly higher than in digester B ($P = 0.035$, student's t-test).

As such, average propionate-to-acetate ratio (3.4 in A vs 1.9 in B) and VFA-to-alkalinity (α) ratio (0.11 in A vs 0.04 in B) were considerably lower in B. The α-ratio reflects the balance, or the lack of it, between the potential cause of pH drop (VFA accumulation) and the buffering capacity of the system (alkalinity) and is commonly used to monitor the system's stability.[33] The lower α ratio and hence improved stability in digester B, can be attributed to the addition of compost and leachate. Stability is a common concern in thermophilic AD of OFMSW whereby meta-stable, rather than fully-stable, conditions often occur with vulnerability to operational disturbances.[33] Note that butyrate was detected only toward the end of the experiment with an average of 21 mg L^{-1} in A, compared to 8 mg L^{-1} in B (Fig. 4(a)).

The alkalinity was comparable in both digesters and started at high concentrations initially (~9000 mg L^{-1} in A and 8000 mg L^{-1} in B), then leveled out to an average of ~5000 mg L^{-1} in both digesters (Fig. 4(b)). However, the intermediate-to-partial alkalinity ratio (IA/PA) and the intermediate-to-total alkalinity ratio (IA/TA)

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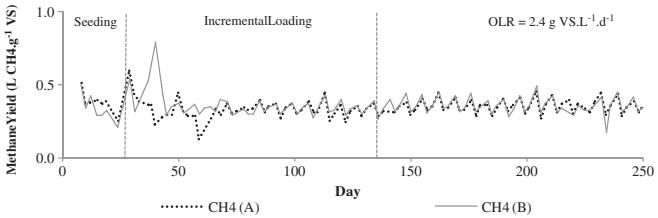

Figure 5. Weekly average of methane generation.

were higher in digester A during startup. The IA/PA and IA/TA ratios are often considered as alternative process stability indicators in thermophilic digesters[21] with process failure thresholds of 0.9 and 0.5, respectively. While IA/PA and IA/TA in digester B remained below 0.6 and 0.4, respectively, they reached 1.0 and 0.5 in digester A by the end of the incremental loading (Fig. 4(c)), equally implying that digester B was more stable during startup.

BIOGAS GENERATION

Throughout the experimental program, both digesters were fed every Monday, Wednesday and Friday and methane generation was reported accordingly (Fig. 5). Thus, the oscillations in Fig. 5 reflect the weekly pattern, whereby methane generation is highest on Mondays (corresponding to the peaks) as a result of an additional resting day on Sunday where VFAs that accumulated during the week were degraded, resulting in peaks of biogas yield with a concomitant increase in pH.

During startup, methane generation was slightly (6%) higher in digester B (366 mL g^{-1} VS compared with 344 mL g^{-1}VS in digester A), similar to reported observations by Neumann and Scherer[12] upon adding compost. However, this difference remains within the margin of error of the measurements and the results can be considered comparable. Similarly, during steady-state, the biogas generation and methane specific yield were close in both digesters, despite differences in the methanogenic composition. Specific methane yields during steady-state reached 360 and 365 L CH_4 g^{-1}VS. in A and B, respectively (Fig. 5), which are within reported ranges (300–400 L CH_4 g^{-1}VS d^{-1}) for thermophilic digesters treating various types of OFMSW at OLR ~2.8 g VS L^{-1} d^{-1}.[35]

Seed impact

Hydrogenotrophs and acetotrophs were dominated by *Methanobacteriales* and *Methanosarcinaceae*, respectively. *Methanomicrobiales* and *Methanosaetaceae* were almost absent in the digesters, despite their considerable relative abundance in both seeds. Accordingly, the seed does not seem to favor a specific methanogenic group. While seeds are reported to have a significant impact on microbial diversity and composition during and after the startup phase,[36,37] operational conditions (digester type, temperature, mixing, OLR and/or HRT) may cause shifts in microbial communities, thus attenuating the impact of the initial seed.[38,39] Also, the elapsed time between the additions of different seeds allowed for microbial acclimation in both digesters, leading to closely similar microbial compositions.[40] Furthermore, the addition of compost (78% acetotrophs) and leachate (94% hydrogenotrophs) did not balance the increase in both types of methanogens in digester B. The hydrogenotrophic methanogens in the leachate consisted almost only of *Methanomicrobiales*, which was not favored under the experimental conditions of this study. On average, the relative abundance ratio of acetotrophs-to-hydrogenotrophs under steady-state conditions was 66-to-34% in digester A and 76-to-24% in digester B, implying a 10% shift toward acetoclastic pathways in B.

Most notably, digester B that was enriched with a mix seed of leachate and compost exhibited enhanced propionate degradation and lower VFA-to-alkalinity (α) ratio (a stability indicator). Also, it demonstrated faster pH stabilization and reduced ammonia levels. Improved propionate degradation in digester B could be due to the improved inoculation strategy and presence of major propionate oxidizers, such as *Syntrophobacter* and *Smithella,* that are lacking in manure and manure-seeded digestate.[28] Further examination of the bacterial composition and diversity may ascertain this postulation.

CONCLUSION

Successful performance of thermophilic anaerobic digesters was attained with a designed mix of non-acclimated seeds based on their methanogen composition, using Quantitative Polymerase Chain Reaction (QPCR) of 16S rRNA gene, to achieve high abundance and diversity of methanogens. Enrichment of digester B with additional seeds, namely leachate and compost, induced a faster acclimation of the microbial flora, better degradation of soluble compounds, enhanced stability manifested by a lower VFA concentration (510 mg L^{-1} in A vs 232 mg L^{-1} in B), mainly propionate, and an increased buffering capacity (α-ratio of 0.11 in A vs 0.04 in B). The relative abundance of acetotrophs was 10% higher in digester B compared with digester A.

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