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Use of lyophilized and acclimated digestate dominated by *Methanobrevibacter* as a start-up inoculum in anaerobic digester led to higher methane production in biochemical methane potential assays

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ABSTRACT

Transporting wet inoculum for full-scale anaerobic digester (AD) start-up is usually infeasible and costly, especially, for remote locations. To overcome these burdens lyophilized AD inoculum is thought to be used after on-site acclimation. For this reason, in this study, the impact of three different acclimated lyophilized AD inoculums collected from full-scale mesophilic AD installations treating different feedstocks was tested for 20 days to monitor AD start-up and methane production by using biochemical methane potential (BMP) assays. The lyophilized inoculums after acclimation were fed to corresponding triplicate digesters treating similar feedstocks as digestate (DG), waste activated sludge (WAS) plus landfill leachate (LL) and WAS, LL plus food waste from municipal solid waste (FWMSW). As a control, no inoculum added digesters with three different feedstocks collected freshly from full-scale mesophilic AD installations treating DG, WAS + LL and WAS + LL + FWMSW were also run in triplicates. All the digesters displayed enhanced methane production in two days of the incubation, the digesters fed with DG as an inoculum displayed shortened start-up and the highest methane production with 42.77 % comparing to control. BMP assays of the other two inoculums tested also displayed 4.73 % enhanced methane production for WAS plus LL and 4.51 % enhanced methane production for WAS, LL plus FWMSW comparing to their corresponding controls. Metagenome analyses of the inoculums used revealed that the dominant methanogens were *Methanobacteriaceae* (100 % *Methanobrevibacter*) for DG, %33 *Methanosaetaceae* (%100 *Methanothrix*) and %27 *Methanobacteriaceae* (%71 *Methanobrevibacter* and %29 *Methanosphaera*) for WAS + LL, %35 *Methanosaetaceae* (%100 *Methanothrix*) and %30 *Methanobacteriaceae* (%91 *Methanobrevibacter* and % 9 *Methanosphaera*) for WAS + LL + FWMSW. The lyophilized DG dominated by hydrogenotrophic genus *Methanobrevibacter* seems to be promising inoculum after acclimation, however, its efficiency needs to be further analysed for the ADs treating various feedstocks.

Introduction

Anaerobic digestion (AD) which is a viable and sustainable waste management tool, is used to treat organic waste and recover energy in the form of methane (Dechrugsa et al., 2013). AD systems have been designed to treat various feedstocks to improve efficiency and methane yield (Khalid et al., 2011). Inoculum usually determines start-up potential of AD and is crucial for stable and long term operations. For this reason one of the key factors that directly influence on biogas yields is selection and use of inoculum that contains appropriate groups of microorganism interacting with each other and able to adapt to various environmental conditions. The success of AD depends on different types of microorganisms that are involved in hydrolysis, acidogenesis, acetogenesis, and methanogenesis. These microorganisms work in synthrophy and any failure to maintain a balance among these microorganisms leads to bioreactor instability (Merlin Christy et al., 2014). Inoculum may also lead to decrease in methanogenic activity and low methane and biogas production (Angelidaki et al., 2009). Therefore, the inoculum from an existing digester treating the same type of substrate and/or exposed to similar conditions usually provides efficient microbial seed for AD. Otherwise, fresh animal manure from ruminants can be used instead but may lead to a longer start-up period (El-Fadel et al., 2013). The microorganisms in manure are mostly anaerobic and are

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Nomenclature

Acronyms

AD	Anaerobic digester
AMPTS	Automatic Methane Potential Test System
BMP	Biochemical methane potential
COD	Chemical oxygen demand
DG	Digestate
DNA	Deoxyribonucleic acid
dsDNA HS	Double stranded DNA high sensivity
FWMSW	Food waste from municipal solid waste
I/S	Inoculum/substrate ratio
LL	Landfill leachate
p	p-value
pH	Potential of Hydrogen
SD	Standard deviation
TKN	Total Kjeldahl nitrogen
TS	Total solids
VS	Volatile solids
WAS	Waste activated sludge

Chemicals

CO_2	Carbon dioxide
H_2S	Hydrogen sulphide
N_2	Nitrogen
NaOH	Sodium hydroxide
O_2	Oxygen
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde

Units

°C	Celsius
%	Percentage
cm	Centimeter
g	Relative centrifugal force
h	Hour
L	Liter
M	Molar
mg	Milligram
min	Minute
mL	Milliliter
mm	Millimeter
mS	MilliSiemens
NmL	Normalised volume
Pa	Pascal
rpm	Revolutions per minute

ideal to decompose the organic material in AD to produce methane (Moset et al., 2014). The use of wet inoculums, however, may be incompatible due to difficulty in handling of large amounts, transportation for long distances and loss of microbial activity during storage. It has been shown that acclimated inoculums provide stability and favour development of AD specific microbial consortium (Lee et al., 2017; Steinmetz et al., 2016). If inoculum is available in a dehydrated, preserved form that can be re-hydrated on-site with minimal additional start-up time, compared to fresh inoculum, start-up of AD in remote or new locations could become more feasible. This study based on the hypothesis of using on-side re-hydrated inoculum to improve the methane production. For this aim, the impact of three different acclimated lyophilized AD inoculums collected from full-scale mesophilic AD installations treating different feedstocks from stable industrial ADs were tested to monitor AD start-up and methane production.

Materials and methods

The experiments were done in three steps: In the first step, AD reactors were run to prepare the inoculums in the lyophilized form and to fix the samples in parallel for later use in microbial analyses. In the second step, prepared inoculums were preserved for a month and then acclimated. In the third step, acclimated inoculums were used to seed the AD reactors to monitor biochemical methane potential. All experiments were performed in triplicate batch AD reactors at mesophilic conditions (37 °C).

Inoculum sources

To prepare the inoculums, three different feedstocks were collected freshly from full-scale mesophilic AD installations treating digestate (DG), waste activated sludge (WAS) plus landfill leachate (LL) and WAS, LL plus food waste from municipal solid waste (FWMSW). Collected feedstocks were kept in 5 L polyethylene vessels at 4 °C. For seeding the feedstocks, fresh dairy cattle manure and landfill leachate (with 8 % TS content) were obtained from 10 L continuously-stirred tank AD under mesophilic conditions (Liu and Lv, 2016). Physiochemical properties of the feedstocks used were determined as pH, conductivity, total solids (TS), volatile solids (VS), chemical oxygen demand (COD), and total Kjeldahl nitrogen (TKN) (APHA, 2012).

Inoculum preparation and characterization

Seeding of feedstocks with 2:1 inoculum/substrate ratio (I/S) based on VS with a total 1.6 L I/S mixture was prepared after depletion any residual biodegradable organic material in the seeds (Gu et al., 2014). Triplicate 2 L AD reactors were run for each seeded feedstocks as DG, WAS + LL, and WAS + LL + FWMSW. An amount of 400 mL headspace in each AD reactor was left for gas accumulation. TS of each AD reactor was adjusted 8 % with distilled water. To assure anaerobic conditions and to eliminate O_2, the reactors were flushed with N_2 gas for 2 min before closing and tightening with plastic screw caps carefully. The AD reactors were placed into a thermostatic water bath and incubated. For CO_2 absorption unit, 2 L of 3 M NaOH solution was prepared and mixed with 0.4 % Thymolphthalein pH indicator solution. The reactors were monitored for 20 days by using biochemical methane potential (BMP) assays. BMP assays were carried out by using The Automatic Methane Potential Test System (AMPTS Iİ Light) (Bioprocess Control, Sweden) and the methane content was recorded in the AMPTS software (AMPTS Light 2.1 v1.2948). At highest methane production depending on BMP results, 400 mL aliquot from each AD reactors was collected and the particulate matter (>1 mm) was removed from the aliquots by passing through sieve to use in fixation and lyophilisation.

Sample fixation for microbial analyses

Fixation of the samples were also done for later use in microbial analyses. For this reason, 6 mL aliquot from each AD reactor was transferred to falcon tubes and centrifuged at 10000 rpm for 5 min. After centrifugation, the supernatant was removed. An amount of 3 mL cold 4 % paraformaldehyde (PFA) was added to the pellet and mixed through vortexing (Inovia, Mini-V2). The tubes were then kept on ice for 4 h before centrifuging at 10000 rpm for 5 min to separate the PFA. In the last step, 3 mL of cold 1:1 phosphate-buffered saline (PBS) and ethanol solution was added to the pellet and mixed. The fixed samples were kept at −20 °C in 1.5 mL Eppendorf tubes until to use in microbial analyses (Nielsen et al., 2009).

Sample lyophilisation to use as inoculums

For preparation of lyophilized inoculums, 10 % skim milk was used as a cryoprotectant (Yarberry et al., 2019). Aliquots from three AD reactors were separately washed in PBS through centrifuging at 10000 rpm for 10 min. The pellets were then re-suspended in PBS and mixed with 10 % skim milk (2.5 mL cryoprotectant/g wet weight pellet). Each mixture was placed in 500-mL round-bottom flask and covered with parafilm to keep at −20 °C for a day. For lyophilisation, the mixture solutions were placed in the Freeze Dryer BK-FD12P (Biobase, China) and lyophilized under 3.7 Pa pressure for 12 days. Lyophilized inoculums were stored at −20 °C for a month until their acclimation to use in BMP assays.

Acclimation of inoculums

As a rehydration solution, a modified nutrient medium with the following ingredients was used for acclimation of inoculums: $CaCl_2$ (0.2 g/L), $MgSO_4$ (0.09767 g/L), KCl (0.4 g/L), NaCl (6.8 g/L), NaH_2PO_4 (0.122 g/L), L-Arginine HCl (0.021 g/L), L-Cysteine 2HCl (0.01565 g/L), L-Glutamine (0.292 g/L), L-histidine (0.008 g/L), L-Isoleucine (0.026 g/L), L-Leucine (0.026 g/L), L-Lysine HCl (0.03647 g/L), L-Methionine (0.0075 g/L), L-Phenilalanine (0.0165 g/L), L-Threonine (0.024 g/L), L-Tryptoptophan (0.004 g/L), L-Tyrosine 2Na2H_2O (0,02595 g/L), L-Valine (0.0235 g/L), D-Biotin (0.001 g/L), Choline Chloride (0.001 g/L), Folic Acid (0.001 g/L), Myo-Inositol (0.002 g/L), Niacinamide (0.001 g/L), D-Pantothenic Acid (hemicalcium) (0.001 g/L), Pyridoxal·HCl (0.001 g/L), Riboflavin (0.0001 g/L), Thiamine·HCl (0.001 g/L), D-Glucose (1 g/L), Phenol Red·Na (0.011 g/L), L-Glutamine (0.292 g/L), $NaHCO_3$ (2.2 g/L). An amount of 20 g lyophilized inoculum from three different sources was separately mixed with 544 mL rehydration solution (>30 % of active reactor volume) and acclimated at mesophilic conditions (37 °C) in 2 L AD reactors for 7 days (Angelidaki et al., 2006). Three different acclimated AD inoculums were selected depending on their initial methane productions were stable.

Biochemical methane potential (BMP) assays

BMP assays were conducted according to the manual of AMPTS II Light (Bioprocess Control, Sweden). AMPTS II system removes biogas CO_2 using an alkaline trap before measuring gas production, and provide cumulative standardized methane volume directly (Fig. 1). The AMPTS II system used in the study contained thermostatic water bath with a capacity for 6x2 L AD reactors. The reactors were prepared depending on ratio: $VS_{sample}/VS_{inoculum} \leq 0.5$) of carbon as substrate (Steinmetz et al., 2016). AD reactors with substrates DG, WAS + LL, and WAS + LL + FWMSW were seeded with the corresponding acclimated inoculums and placed in the thermostatic water bath. As a control, no inoculum added digesters with three different substrates collected freshly from full-scale mesophilic AD installations treating DG, WAS + LL and WAS + LL + FWMSW were also run. Each AD reactor was connected to a mechanical agitator to provide gentle mixing for feedstock and inoculum. The gas produced in the AD reactors was transferred to the corresponding scrubbing unit (CO_2 absorption tray) through tubes. Alkali solution within the scrubbing unit removed CO_2 or H_2S produced during anaerobic digestion allowed methane to pass to the gas volume measuring device during which the data were recorded by AMPTS software (AMPTS Light 2.1 v1.2948).

Metagenomic analysis

For metagenome analysis, 1.5 mL samples were taken from each inoculum sources as DG, WAS + LL, and WAS + LL + FWMSW. Upon sampling, they were centrifuged at 15000 rpm at 4 °C for 10 min. The supernatants were discarded and the pellets were stored at −20 °C for subsequent DNA extraction. During DNA extraction the instruction from PowerSoil® DNA Isolation Kit (Mo Bio, USA) was followed. DNA purity was assessed with a NanoDrop One spectrophotometer (Thermo Fisher Scientific, USA), and for DNA quantification, the Qubit ™ dsDNA HS Assay Kit (Thermo Fisher Scientific, USA) was used. DNA samples were sent to the Genome Quebec Innovation Centre (Montreal, Canada), where the shotgun metagenomic sequencing was performed in an Illumina MiSeq PE250 platform.

Statistical analyses

Data were presented as average ± standard deviation (SD) from triplicate. Statistical differences between data sets were verified by ANOVA at $p < 0.05$.

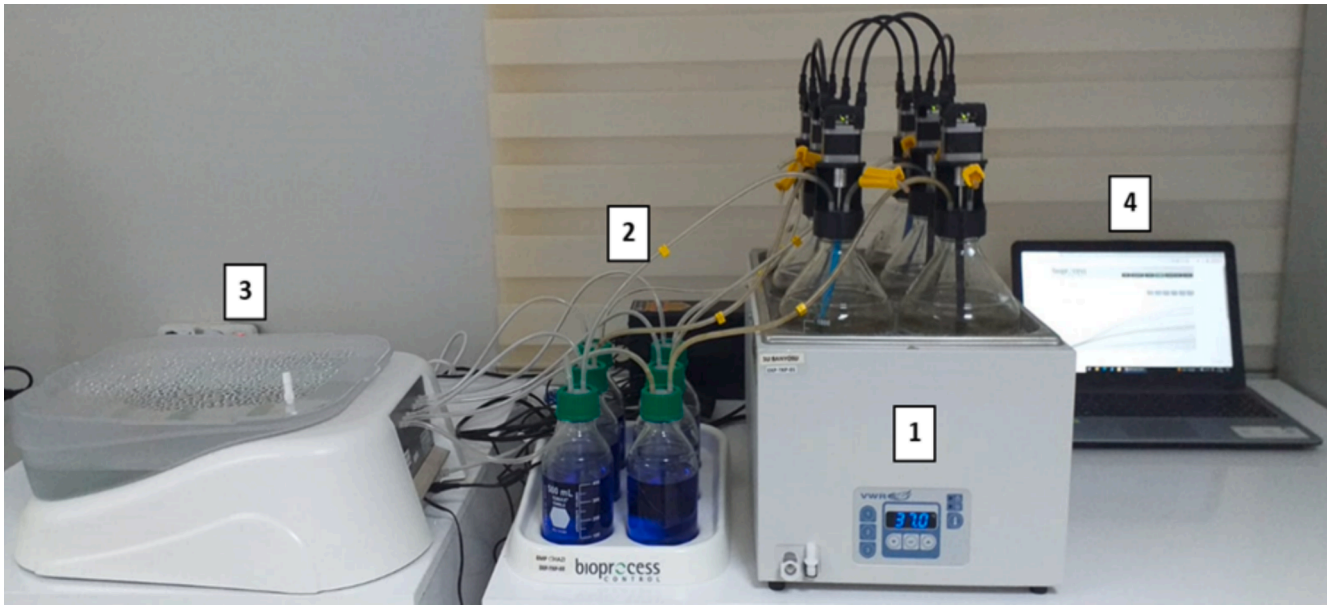

Fig. 1. AMPTS II system used in the study: 1, Thermostatic water bath; 2 CO_2 absorption tray; 3 gas volume measuring device and 4 AMPTS software (AMPTS Light 2.1 v1.2948).

Results and discussion

BMP assays with acclimated inoculums

Start-up inoculum volume usually ranges from 10 to 60 % of total AD reactor volume (Angelidaki et al., 2006; Ike et al., 2010). For industrial AD reactors, this leads to using more than thousands cubic meter of inoculum for the initiation of the digestion process. Considering the geographical location of AD plants, this requirement costs a lot and possibly becomes infeasible due to use of fresh inoculums. Dehydrated and preserved inoculum for initiation of AD and using it in rehydrated form on-site might overcome these burdens. For this reason in this study, the efficiency of lyophilized inoculum after acclimation for digester start-up and methane yield were investigated. For the preparation of lyophilized inoculums ADs with the feedstocks of DG, WAS plus LL and WAS, LL plus FWMSW were used as the inoculum sources. Varying inoculum sources lead to changes in substrate adaptation and biodegradability (Yang et al., 2017). The source of inoculum also affect degradation rate/time, biogas composition, and reactor stability (Gao et al., 2018). Therefore, the inoculums prepared in this study were used for seeding the ADs treating the same type of feedstock. Physiochemical properties of the feedstocks were determined (Table 1). The pH was ranging from 4.5 (in FWMSW) to 7.7 (in WAS). The highest conductivity was observed in DG (35.64 mS/cm) and LL (28.68 mS/cm). TS and VS were higher in WAS (20.97 % and 10.40 %, respectively) and FWMSW (35.03 % and 18.25 %, respectively). However, CODs of DG (37950 mg/L) and WAS (30784 mg/L) were higher comparing to other feedstocks. DG also displayed the highest TKN value (11043 mg/L). The highest C/N was calculated for FWMSW.

Collected feedstocks with almost 8 % TS content were used to prepare DG, WAS plus LL and WAS, LL plus FWMSW inoculums as suggested by Liu and Jian (2016). Physiochemical properties of the inoculums prepared were also determined (Table 2). The pH of all inoculum solutions was in between 7–8. Conductivity was measured approximately 25 mS/cm. VS of the inoculum solutions did not show any significant variations (4–5 %). Great variations were observed in COD and TKN. Although DG and WAS + LL inoculum solutions displayed similar C/N of 8 %, this ratio was almost 15 % for WAS + LL + FWMSW. C/N ratio that varies with feedstock type is an important parameter during AD process (Yan et al., 2015). The high COD and TKN values of feedstock seemed to be lowered while preparing inoculum solutions. These inoculum solutions were lyophilized for long term preservation. After a month, lyophilized inoculums were acclimated to use in BMP assays.

Acclimated inoculums were used to seed corresponding AD reactors treating the same type of feedstock as DG, WAS + LL, and WAS + LL + FWMSW. As a control, AD reactors without inoculums were also run for each. Initial and final physiochemical properties of the control and seeded reactors were also determined (Table 3). The pH of all the reactors was varying in between 7.14 and 7.91. Final pH values were higher than the initial ones for all the reactors. The pH levels lower than 6.0 and higher than 8.5 are reported to be toxic for methanogens (Chandra et al., 2012; Zhao et al., 2013).

Table 1
Physicochemical characteristics of the feedstocks used in the study.

	pH	Conductivity (mS/cm)	TS (%)	VS (%)	COD (mg/L)	TKN (mg/L)
DG	7.41 ± 0.01	35.64 ± 0.02	8.81 ± 1.00	5.28 ± 1.00	37950 ± 40.00	11043 ± 18.00
LL	5.97 ± 0.02	28.68 ± 0.01	2.30 ± 0.00	0.89 ± 0.00	5824 ± 6.00	2063 ± 1.00
WAS	7.70 ± 0.02	2.93 ± 0.01	20.97 ± 1.00	10.40 ± 1.00	30784 ± 7.00	3230 ± 5.00
FWMSW	4.53 ± 0.01	6.17 ± 0.02	35.03 ± 0.00	18.25 ± 0.00	25792 ± 5.00	510 ± 3.46

DG, digestate; WAS, waste activated sludge; LL, landfill leachate; FWMSW, food waste from municipal solid waste;
TS, total solids; VS, volatile solids; COD, chemical oxygen demand; TKN, total Kjeldahl nitrogen; ± standart deviations.

Table 2
Physicochemical characteristics of the inoculums prepared.

	pH	Conductivity (mS/cm)	TS (%)	VS (%)	COD (mg/L)	TKN (mg/L)
DG	7.39 ± 0.04	24.45 ± 0.01	7.76 ± 0.07	4.78 ± 0.15	32850 ± 2192	4190 ± 14
WAS + LL	7.87 ± 0.02	22.94 ± 0.02	7.77 ± 0.03	4.46 ± 0.08	44350 ± 1626	5580 ± 56
WAS + LL + FWMSW	7.85 ± 0.02	24.84 ± 0.02	7.28 ± 0.11	4.01 ± 0.02	69225 ± 601	4730 ± 57

DG, digestate; WAS, waste activated sludge; LL, landfill leachate; FWMSW, food waste from municipal solid waste;
TS, total solids; VS, volatile solids; COD, chemical oxygen demand; TKN, total Kjeldahl nitrogen; ± standart deviations.

The pH values obtained for all reactors were within the optimum range. This was also confirmed with low C/N ratio obtained from the reactors. The highest C/N ratio was obtained as 12 % for WAS + LL + FWMSW. Although the optimal C/N ratio for ADs ranges in between 20/1 and 30/1, this ratio varies depending on feedstock type (Yan et al., 2015). All the seeded reactors showed higher removal for the parameters tested comparing to their corresponding controls. The highest decrease in conductivity (34 %) was observed in WAS + LL + FWMSW. Initially settled TS values (8 %) dropped to 6.35–7.15 %. The highest TS (almost 21 %), VS (almost 38 %) and TKN (almost 54 %) removals were obtained in DG. The rate of VS removal reflects to biodegradable organic matter conversion and biogas/methane production in AD (Yang et al., 2017). Therefore, the highest removal rate of VS obtained in DG was thought to be the sign of better efficiency. Similar trend was not observed in COD removals. The COD removal was ranged in between 18 % (in DG) and 30 % (in WAS + LL + FWMSW). During AD, COD is converted to methane and carbon dioxide. Therefore, COD removal efficacy can also be used to assess methane yield.

Cumulative methane production was also determined by using BPM assays (Table 4). VS unit reflects the efficiency of biodegradable organic matter conversion as indicated by Yang et al., (2017). The increase in cumulative methane production for DG, WAS plus LL, and WAS, LL plus FWMSW comparing to their corresponding controls were 22.69, 11.34 and 12.25 NmL/gVS, respectively. Similarly, cumulative methane production (in NmL) for DG (42.77 %) was higher than for WAS plus LL (4.73 %), and WAS, LL plus FWMSW (4.51 %) comparing to their corresponding controls (Fig. 2). Cumulative methane productions in both NmL and NmL/gVS were found to be significant for all seeded reactors comparing to their corresponding controls ($p < 0.05$).

AD start-up and archaeal community analyses

In terms of AD start-up, no difference was determined for WAS plus LL and WAS, LL plus FWMSW comparing to their corresponding controls (Fig. 2). However, methane production for DG started earlier than its control. These results confirmed the hypothesis that lyophilized and acclimated inoculum from an existing digester treating the same type of feedstock provided efficient microbial seed for AD. Although all the seeded reactors started to produce methane on the second day, their peak values were obtained on 4th day as 1166.8NmL for DG, on 5th and 6th days as 1333.1 and 1510.4 NmL for WAS plus LL and WAS, LL plus

Table 3
Initial and final physiochemical properties of the seeded and unseeded reactors.

	pH		Conductivity (mS/cm)		TS (%)		VS (%)		COD (mg/L)		TKN (mg/L)	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
Control	7.71 ± 0.02	7.91 ± 0.00	17.70 ± 0.01	14.10 ± 0.06	8.00 ± 0.01	6.40 ± 0.02	7.17 ± 0.02	4.55 ± 0.02	43400 ± 43.30	38300 ± 18.03	8502 ± 10.06	6951 ± 17.78
DG	7.70 ± 0.01	7.88 ± 0.02	17.77 ± 0.02	13.42 ± 0.03	8.00 ± 0.00	6.35 ± 0.03	7.18 ± 0.00	4.46 ± 0.01	43860 ± 37.75	36100 ± 45.83	8630 ± 8.66	4022 ± 23.58
Control	7.28 ± 0.02	7.56 ± 0.00	19.13 ± 0.03	16.95 ± 0.02	8.11 ± 0.01	6.82 ± 0.01	7.02 ± 0.05	5.13 ± 0.04	76400 ± 52.92	62200 ± 36.06	9792 ± 11.14	8956 ± 17.52
WAS + LL	7.23 ± 0.00	7.90 ± 0.01	19.08 ± 0.01	16.03 ± 0.02	8.10 ± 0.00	7.15 ± 0.04	7.04 ± 0.01	5.47 ± 0.28	76120 ± 21.79	59800 ± 20.00	9860 ± 15.52	8102 ± 20.65
Control	7.14 ± 0.02	7.59 ± 0.01	23.05 ± 0.04	17.25 ± 0.00	8.17 ± 0.05	6.70 ± 0.03	6.40 ± 0.02	4.75 ± 0.01	78820 ± 26.46	64700 ± 55.67	6134 ± 17.44	6027 ± 26.65
WAS + LL + FWMSW	7.16 ± 0.00	7.63 ± 0.00	23.12 ± 0.03	15.07 ± 0.01	8.15 ± 0.00	6.94 ± 0.02	6.43 ± 0.00	4.95 ± 0.01	78890 ± 35.00	55400 ± 26.46	6245 ± 18.03	5890 ± 16.62

DG, digestate; WAS, waste activated sludge; LL, landfill leachate; FWMSW, food waste from municipal solid waste; TS, total solids; VS, volatile solids; COD, chemical oxygen demand; TKN, total Kjeldahl nitrogen; ± standart deviations.

Table 4
BMP assay results.

	Cumulative Methane	
	(NmL)*	(NmL/gVS)*
Control	3496.0 ± 6.65	45.84
DG	6108.9 ± 19.32	68.53
Control	8705.6 ± 5.35	136.59
WAS + LL	9137.9 ± 17.97	147.93
Control	9903.3 ± 4.79	168.04
WAS + LL + FWMSW	10370.6 ± 20.74	180.30

DG, digestate; WAS, waste activated sludge; LL, landfill leachate FWMSW, food. waste from municipal solid waste; ± standart deviations; *, significance level of $p < 0.05$.

FWMSW, respectively. DG seeds seemed to shorten start-up time and enhance cumulative methane production even the peak methane production value was lower than the other seeds. This was accounted for not only DG itself but also the microbial flora in it. Therefore, in order to analyse the efficacy of each seeds in methane yield, microbial community analyses of the seeds, especially in terms of archaea, was also done.

The taxonomic distribution of the microbial community indicated that the most abundant domain was *Bacteria* (98–99 %) followed by *Archaea* (1–2 %). The analyses also revealed that the percentages of the archaeal community were 1.26 % for DG, 1.56 % for WAS plus LL, and 2 % for WAS, LL plus FWMSW. In the archaeal community, the most abundant families were *Methanobacteriaceae* (100 % *Methanobrevibacter*) for DG, %33 *Methanosaetaceae* (%100 *Methanothrix*) and %27 *Methanobacteriaceae* (%71 *Methanobrevibacter* and %29 *Methanosphaera*) for WAS + LL, %35 *Methanosaetaceae* (%100 *Methanothrix*) and %30 *Methanobacteriaceae* (%91 *Methanobrevibacter* and %9 *Methanosphaera*) for WAS + LL + FWMSW (Fig. 3).

Other archaeal families within WAS plus LL, and WAS, LL plus FWMSW were also determined. WAS + LL also contained the archaeal families %23 *Methanomicrobiaceae* (%91 *Methanoculleus* and %9 *Methanolacinia*), %11 *Methanosarcinaceae* (%100 *Methanosarcina*), %5 *Halorubraceae* (%100 *Halorubrum),* and %1 *Methanospirillaceae* (%100 *Methanosprillum*). Other archaeal families determined in WAS + LL + FWMSW were %18 *Methanomicrobiaceae* (%91 *Methanoculleus* and %9 *Methanomicrobium*), %16 *Methanosarcinaceae* (%100 *Methanosarcina*), % 1 *Methanocorpusculaceae* (%100 *Methanocorpusculum*). All the tree inoculums prepared contained the archaeal familiy *Methanobacteriaceae* with the dominant genus *Methanobrevibacter* (hydrogenotrophic) in common. Apart from DG, WAS + LL and WAS + LL + FWMSW inoculums also had the genus *Methanosphaera* within the same family. The methanogenic family *Methanobacteriaceae* (order *Methanobacteriales*, class *Methanobacteria*) includes four genera: *Methanobacterium*, *Methanobrevibacter*, *Methanosphaera* and *Methanothermobacter*. They obtain energy from the reduction of CO_2 with H_2. *Methanosphaera* spp. do not reduce CO_2 but obtain their energy only from the reduction of methanol by H_2 (Oren, 2014). Both inoculums of WAS + LL and WAS + LL + FWMSW also contained the archaeal family *Methanosaetaceae* with the dominant genus *Methanothrix*. Acetate is the most important precursor of methane production and up to 70 % of methane is yielded over acetate in ADs. *Methanosaeta* (formerly known as *Methanothrix*) and *Methanosarcina* that are methanogenic genera contain spp. that are able to utilize acetate (acetoclastic). Apart from acetoclastic activity, *Methanosarcina* spp. are also capable of using methanol, methylamines and sometimes H_2 and CO_2 as growth substrates, while *Methanosaeta* spp. are restricted to growth only on acetate (Anderson et al., 2003). At mesophilic conditions methane is always produced by a combination of acetoclastic methanogenesis involving *Methanosarcinaceae, Methanosaetaceae*, and hydrogenotrophic methanogenesis involving

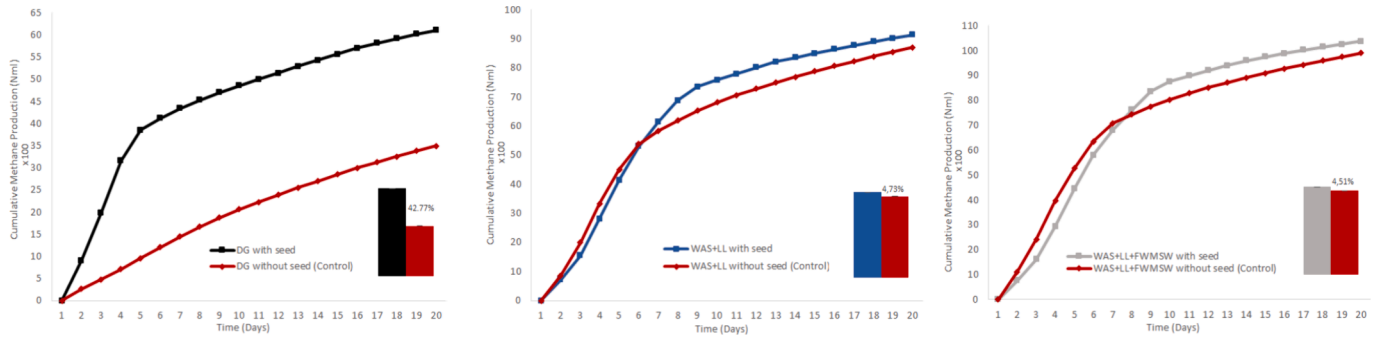

Fig. 2. Cumilative methane production in NmL.

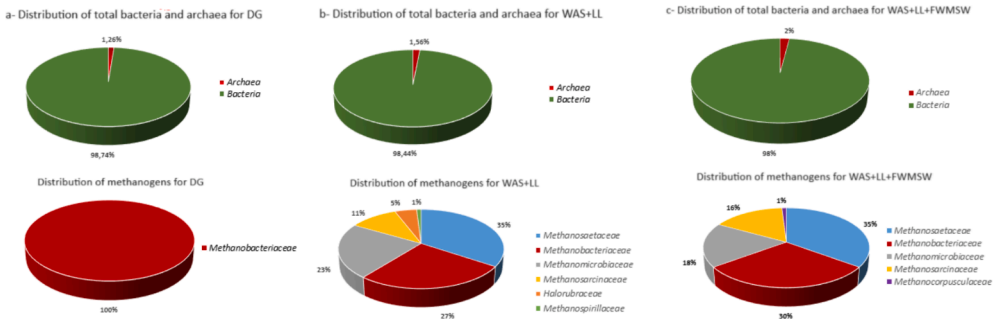

Fig. 3. Distribution of microbial community in the seeds.

Methanomicrobiales, Methanobacteriales, and *Methanocellales* as indicated by Liu and Conrad, (2010). Current study revealed that although DG inoculum included only hydrogenotrophic methanogens, the other two inoculums contained both hydrogenotrophic and acetoclastic methanogens together. DG inoculum dominated with hydrogenotrophic *Methanobrevibacter* since DG had high TKN value (Table 1). Although nitrogen is crucial for microorganisms when it is in high concentration considered to be toxic (Capson-Tojo et al., 2020). The high TKN is an important evidence for the dominance of hydrogenotrophic *Methanobrevibacter* in DG and for the reduction in the relative abundance of the other methanogens. This was in correlation with the finding of Capson-Tojo and his co-authors who found that hydrogenotrophic methanogens were much more resistant to ammonia inhibition than acetoclastic methanogens (Wang et al., 2016). High TKN values have been linked to *Methanobrevibacter* dominance in previous studies as well (Bayrakdar et al., 2017, Molaey et al., 2018). Well-balanced interaction between microorganisms and the feedstock is crucial for the efficiency of methane production in AD (Karakashev et al., 2006; Mata-Alvarez et al., 2014). This was also confirmed in the current study that each inoculum yielded higher methane production when they were seeded ADs treating the same type of feedstock. Therefore, the use of an acclimated inoculums prepared from the same type feedstock improves the efficiency of methane production by favouring the development of specific microbiota as indicated by Lee et al., (2017). The results also showed the potential of using lyophilized DG inoculum dominated by *Methanobrevibacter* for full-scale AD start-up or re-activation in remote locations without additional transportation costs. Compared to wet inoculum transportation costs, lyophilized and on-site acclimated DG inoculums dominated by *Methanobrevibacter* may present cost savings providing that scale-up studies conducted beforehand.

Conclusions

The study indicated that use of lyophilized and acclimated inoculums from an existing digester treating the same type of feedstock provided efficient microbial seed for shorten start-up and enhanced methane production in ADs. Among DG, WAS + LL and WAS + LL + FWMSW inoculums, DG dominated by hydrogenotrophic methanogen *Methanobrevibacter* seemed to be the most promising inoculum, however, scale-up studies of the lyophilized and acclimated inoculums need to be done for full-scale AD installations in remote locations. The results obtained in this study also highlights an alternative use of DG as a tool for cleaner energy production apart from being widely used as fertilizer.

CRediT authorship contribution statement

Deniz Cam: Visualization, Methodology, Investigation, Data curation. **Sena Sayin:** Methodology, Investigation. **Oral Zeki Sarman:** Visualization, Validation, Project administration, Methodology, Conceptualization. **Erol Iren:** Visualization, Validation, Supervision, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization. **Bulent Icgen:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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