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Microbial dynamics in anaerobic digestion reactors for treating organic urban residues during the start-up process

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Abstract

Anaerobic digestion of organic residues offers economic benefits via biogas production, still methane (CH_4) yield relies on the development of a robust microbial consortia for adequate substrate degradation, among other factors. In this study, we monitor biogas production and changes in the microbial community composition in two semi-continuous stirred tank reactors during the setting process under mesophilic conditions (35°C) using a 16S rDNA high-throughput sequencing method. Reactors were initially inoculated with anaerobic granular sludge from a brewery wastewater treatment plant, and gradually fed organic urban residues (4·0 kg VS m^{-3} day^{-1}) . The inocula and biomass samples showed changes related to adaptations of the community to urban organic wastes including a higher relative proportion of Clostridiales, with *Ruminococcus* spp. and *Syntrophomonas* spp. as recurrent species. *Candidatus* Cloacamonas spp. (Spirochaetes) also increased from ~2·2% in the inoculum to >10% in the reactor biomass. The new community consolidated the cellulose degradation and the propionate and amino acids fermentation processes. Acetoclastic methanogens were more abundant in the reactor, where *Methanosaeta* spp. was found as a key player. This study demonstrates a successful use of brewery treatment plant granular sludge to obtain a robust consortium for methane production from urban organic solid waste in Mexico.

Significance and Impact of the Study

This study describes the selection of relevant bacteria and archaea in anaerobic digesters inoculated with anaerobic granular sludge from a brewery wastewater treatment plant. Generally, these sludge granules are used to inoculate reactors digesting organic urban wastes. Though, it is still

not clearly understood how micro-organisms respond to substrate variations during the reactor start-up process. After feeding two reactors with organic urban residues, it was found that a broader potential for cellulose degradation was developed including Bacteroidetes, Firmicutes and Spirochaetes. These results clarify the bacterial processes behind new reactors establishment for treating organic wastes in urban areas.

Keywords: bioprocessing, fermentation biotechnology, metagenomics, sludge, solid waste.

Introduction

During the last decades, international and national development programs have incorporated sustainable policies for social and economical growth; especially in urban areas, where waste management has emerged as an important topic closely related and integrated to develop climate change strategies (Eriksson *et al*. 2014). The anaerobic digestion of organic waste offers major environmental and economic benefits including: production of biogas (with 50–80% methane v/v) from nonfossil sources, retention of gases with greenhouse effect, reduced landfilling requirements, elimination of pathogenic micro-organisms, reduction of odours and flies, and the co-production of a digestate (from sludge) with high fertilization capacity (Holm-Nielsen *et al*. 2009). The success of anaerobic digestion processes relies on the synergic interaction of micro-organisms that decompose organic matter polymers to smaller molecules, including hydrolysing, fermenting, acidogenic and methanogenic micro-organisms (Godon *et al*. 1997; Rivière *et al*. 2009). Consortia can be obtained from several sources such as anaerobic sludge from municipal or industrial wastewater treatment plants, rumen and animal manures, soil extracts and anaerobic sediments (Cardinali-Rezende *et al*. 2010). Generally, sludge granules from anaerobic wastewater treatment plants are used to inoculate reactors digesting urban organic wastes, mainly because they are widely distributed in these areas and have common features among them.

In Mexico, nearly 52% of the urban solid wastes consist of organic residues (~21·5 million tons in 2011) (Durán-Moreno *et al*. 2013; Semarnat 2013). The treatability of this organic portion by anaerobic digestion has been demonstrated (Monroy *et al*. 2000; Garcia-Peña *et al*. 2011); but to date, there are only a few reports investigating the microbial composition of candidate inocula in the area and its changes during the reactor start-up process (Monroy *et al*. 2000).

Even though the microbial community composition of anaerobic digestion has been long studied via culture-dependent and -independent molecular methods (Toerien and Hattingh 1969; Godon *et al*. 1997; Rivière *et al*. 2009; Wilkins *et al*. 2015), most of these studies characterize the bacterial component in well-established reactors. It is still not clearly understood how microbes respond to substrate variations during the reactor start-up and working processes. In Mexico, breweries are a main source of income to the

economy, and although anaerobic treatment plants have been in use in Mexico since the late 1980's, industrial effluents from breweries represent 25% of the anaerobic reactors (Monroy *et al*. 2000). Here, we analyse the potential for biogas production and microbial community composition in anaerobic stirred tank reactors fed urban organic waste. The reactors were inoculated with anaerobic granular sludge from a brewery wastewater treatment plant in Mexico City and monitored during the setting process.

Results and discussion

Reactors and methane production

Bacterial consortia dynamics were observed in two semi-continuous stirred tank reactors (CSTR) under mesophilic conditions (35°C). Both anaerobic digesters contained exclusively granular sludge at the star-up time and the organic residues were gradually incorporated at an organic load of 4·0 kg VS m^{-3} day^{-1} (Fig. 1a). Samples from the effluents were collected to characterize the bacterial and archaeal composition (Fig. 1b). Higher biogas yields were visible after 20 days, considering that both CSTRs operated with a hydraulic retention time of 13 days. In a similar way, volatile solids decreased with time (Table 1, Fig. 2a). The mean concentration of methane in the produced biogas was 59·95%.

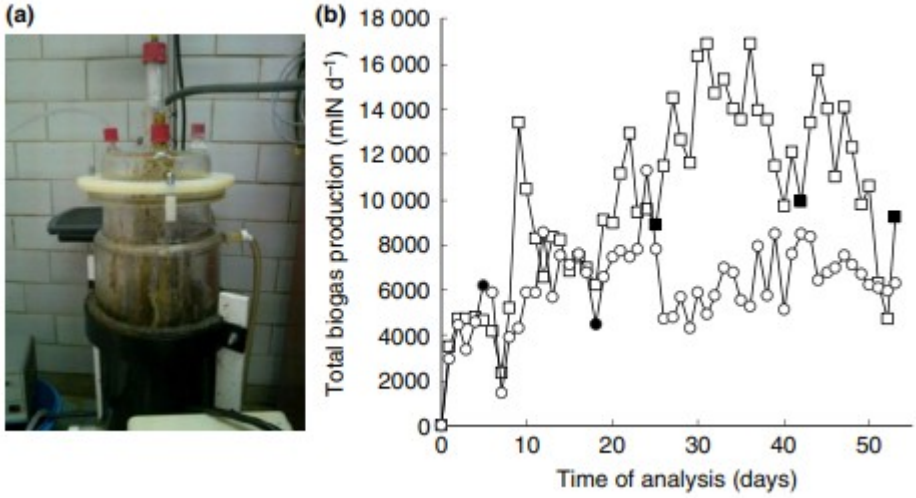

Figure 1. Laboratory-scale anaerobic continuous stirred tank reactors (CSTR) for measuring biogas production during microbial degradation of municipal organic waste. (a) cylindrical-5L semi-continuous stirred tank reactor used during the experiments. (b) Biogas production in two reactors under similar conditions, (○) CSTR-1 and (□) CSTR-2. Symbols in black show biomass sampling points.

Table 1 pH and solids content of inoculum and effluent samples during continuous stirred tank reactors (CSTR) operation

Sample Id	Time of analysis (day)	pH	Alkalinity α	Total solids (g l^{-1})	Volatile solids (g l^{-1})
CSTR-1 (R1)					
R1-t0	0	7·54	0·72	75·30	59·98
R1-t26	26	8·54	0·79	60·33	38·92
R1-t47	47	7·62	0·69	62·29	31·53
R1-t52	52	7·43	0·66	61·20	30·04
CSTR-2 (R2)					
R2-t0	0	7·52	0·72	75·96	61·03
R2-t5	5	7·44	0·70	75·77	59·84
R2-t20	20	8·09	0·76	70·64	41·59

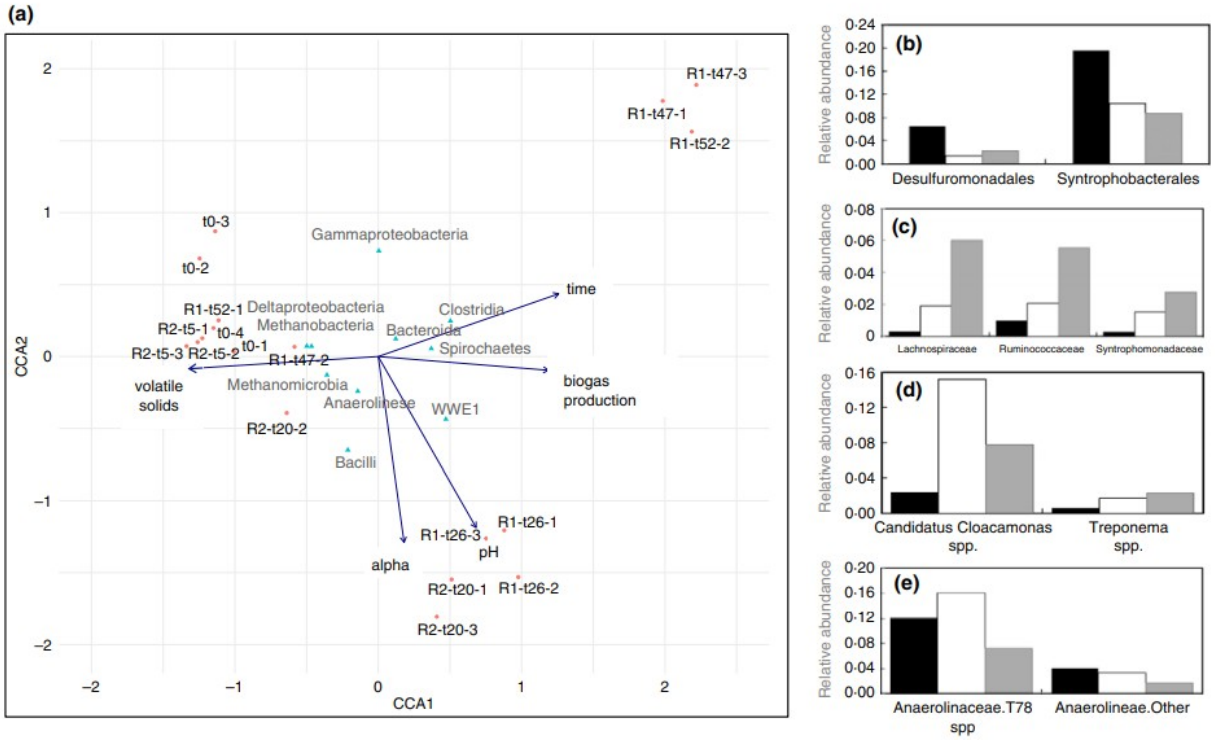

Figure 2. (a) Canonical correspondence analysis (CCA) to visualize differences between the community composition (at class level) in sampling times and continuous stirred tank reactors (CSTR) physicochemical environment. Mean relative proportions in the bacterial classes (b) Deltaproteobacteria; (c) Clostridia; (d) Spirochaetes and (e) Chloroflexi; and its changes at 0–5 days (black bars), 20–26 days (white bars) and 47–52 days (grey bars) of CSTRs establishment.

Development of urban waste degrading microbial consortia

A total of 609 002 sequences of 16S rRNA gene encompassing Bacteria and Archaea were obtained from the inoculum and different time points during the operation of the CSTRs (Fig. 1b). Bacteria represented nearly 96% of the microbial communities of the inoculum while Archaea only 4%, with <1% of unclassified sequences. A similar Bacteria/Archaea proportion was found in all samplings (Table 2). This invariable proportion through time was probably due to the degradation of organic matter, which requires several specialized enzymatic pathways to process carbohydrate polymers, lipids and proteins, most of them found in Bacteria (Gujer and Zehnder 1983).

Table 2 Percent of main bacterial and archaeal phyla in continuous stirred tank reactors (CSTRs). Numbers correspond to averages calculated from replicates, (*) the standard deviation

		CSTR-2 (R2)		CSTR-1 (R1)		
Domain Phyla	Inoculum, sludge, 0 days	5 days	20 days	26 days	47 days	52 days
Bacteria	95·61 (5·86)*	94·18 (5·86)	96·44 (5·03)	96·53 (5·27)	97·82 (5·66)	95·09 (5·19)
Proteobacteria	32·77 (2·56)	33·22 (0·67)	15·76 (6·04)	10·97 (0·07)	15·24 (7·68)	17·07 (15·02)
Bacteroidetes	15·69 (1·93)	14·19 (0·25)	15·49 (1·81)	16·78 (0·92)	23·96 (4·84)	21·98 (4·71)
Chloroflexi	16·33 (2·27)	16·25 (1·10)	20·60 (1·05)	18·24 (0·78)	8·42 (6·33)	10·08 (4·42)
Spirochaetes	4·54 (0·50)	4·51 (0·39)	13·17 (3·98)	22·16 (1·74)	13·42 (4·71)	6·77 (1·93)
Firmicutes	4·78 (2·32)	5·70 (0·17)	10·78 (3·29)	12·42 (1·34)	22·82 (11·13)	19·25 (12·99)
Synergistetes	5·51 (1·04)	7·49 (0·58)	9·93 (2·33)	5·99 (0·26)	5·47 (1·66)	6·75 (0·21)
Others	15·99 (0·61)	12·82 (1·16)	10·71 (2·89)	9·99 (0·32)	8·50 (4·62)	13·18 (0·43)
Archaea	3·61 (0·80)	5·50 (0·24)	2·69 (0·52)	2·88 (0·48)	1·27 (0·44)	2·58 (1·88)
Euryarchaeota	3·54 (0·80)	5·13 (0·21)	2·64 (0·57)	2·97 (0·46)	1·123 (0·40)	2·50 (1·81)
Unclassified	0·78 (0·30)	0·61 (0·13)	0·87 (0·21)	0·59 (0·15)	0·90 (0·49)	2·33 (1·90)

Changes in the bacterial community composition were observed at lower classification levels in response to the addition of organic waste and time of establishment (Table 2, Fig. 2a). The biomass composition was similar at phyla level between 0 and 5 days of operation; but sizable changes were observed in both reactors after that period (Table 2, Fig. 2a). Previous characterization of the organic wastes from central Mexico determined that gardening, vegetable and fruit residues accounted for 63–70% of the organic solid waste sources (Monroy *et al*. 2000). At 0 and 5 days, there was a higher proportion of volatile solids in both reactors, and diminished with the addition of organic wastes (i.e. feeding time) and biogas production (Fig. 2a). As a result, cellulose degradation emerges as a key step for degradation of these urban wastes. Therefore, we focused on understanding the changes in community structure of bacteria that can utilize cellulose and its degradation products. In accordance to the trends observed in biogas production, the major changes in the bacterial genetic diversity were found after 20 days of operation. Proteobacteria diminished from 33% to 10–16%, while Spirochaetes and Firmicutes at least doubled their proportion after (Table 2).

Proteobacteria was the most abundant phylum in the granular sludge (~33%), with Deltaproteobacteria as the main group (~73%), followed by Gammaproteobacteria. Syntrophobacterales and Desulfuromonadales (Deltaproteobacteria) decreased from 24·5% in the inoculum to 11% after the addition of organic wastes during 52 days. Desulfuromonadales was

almost lost from some samples in the bioreactor, whereas Syntrophobacterales species were retained (Fig. 2b). Mesophilic syntrophic propionate-oxidizing species have been described within Deltaproteobacteria, and play an important role as propionate is an abundant fatty acid intermediate in methanogenesis. *Syntrophobacter* spp. was found in both reactors (1–12%). Species of these genus can use sulphate as an electron acceptor and oxidize propionate, and have been previously isolated from methanogenic enrichments and anaerobic reactors (Chen *et al*. 2005).

Orders belonging to Clostridia (within Firmicutes) were notably affected by the addition of organic wastes (Fig. 2c), duplicating its proportion from 5% to ≥10% after 20 days. Clostridiales have also been described as common synthrophs of metanogenic Archaea (Kimura *et al*. 2010). Families Lachnospiraceae, Ruminococcaceae and Syntrophomonadaceae were the most abundant (Fig. 2c), with *Ruminococcus* spp. and *Syntrophomonas* spp. as recurrent species. Ruminocci are cellulolytic bacteria often found in rumen and the human gut, and contain complex cellulosomes with a large number of fibre-degrading enzymes (Berg Miller *et al*. 2009). Complementary, *Syntrophomonas* spp. can oxidize different fatty acids syntrophically with hydrogen/formate-using micro-organisms, such as methanogens (McInerney *et al*. 2008).

Candidatus Cloacamonas spp. (class WWE1) also showed a clear relationship with the addition of urban organic residues and biogas production, increasing its abundance from ~2·2% in the inoculum to >10% in the reactor biomass (Fig. 2a,d). Class WWE1 was firstly detected in sludge samples from an anaerobic mesophilic digester (33°C) at a wastewater treatment plant, but is widespread in anaerobic digesters and are involved in cellulose degradation and/or the uptake of fermentation products (Chouari *et al*. 2005; Limam *et al*. 2014). *Candidatus* Cloacamonas acidaminovorans is a syntrophic bacterium able to ferment amino acids and produce H_2 (Pelletier *et al*. 2008). Other members of phylum Spirochaetes were also related to biogas production (Fig. 2a).

Chloroflexi showed slight variations in their relative proportions during the 26 first days of operation (16–20%), and subsequently presented a small decrease. Interestingly, this group was mainly composed by the T78 group (Fig. 2e). Methane seeps in Arctic permafrost have a stable composition of bacteria and archaea, in which the Chloroflexi group T78 is a dominant member (Lamarche-Gagnon *et al*. 2015).

Anaerobic consortia contain several types of micro-organisms that hydrolyse the organic matter into low-molecular weight compounds such as acetate, CO_2 and H_2 to be later transformed by methanogenic archaea (Gujer and Zehnder 1983). *Methanosaeta* spp. was the main archaeon in the granular sludge (40 ± 24%) (Fig. 3a). This acetoclastic organism represents an important methanogen in mesophilic reactors (McMahon *et al*. 2004; Rivière

et al. 2009). Besides its activity, this filamentous organism is a structural key member in the formation and stability of granular sludge (Hulshoff Pol *et al*. 2004). Acetoclastic methanogens are usually more abundant than hydrogenotrophic methanogens (Díaz *et al*. 2006), and through the process, strict acetoclastic methanogens dominated both reactors (Fig. 3b).

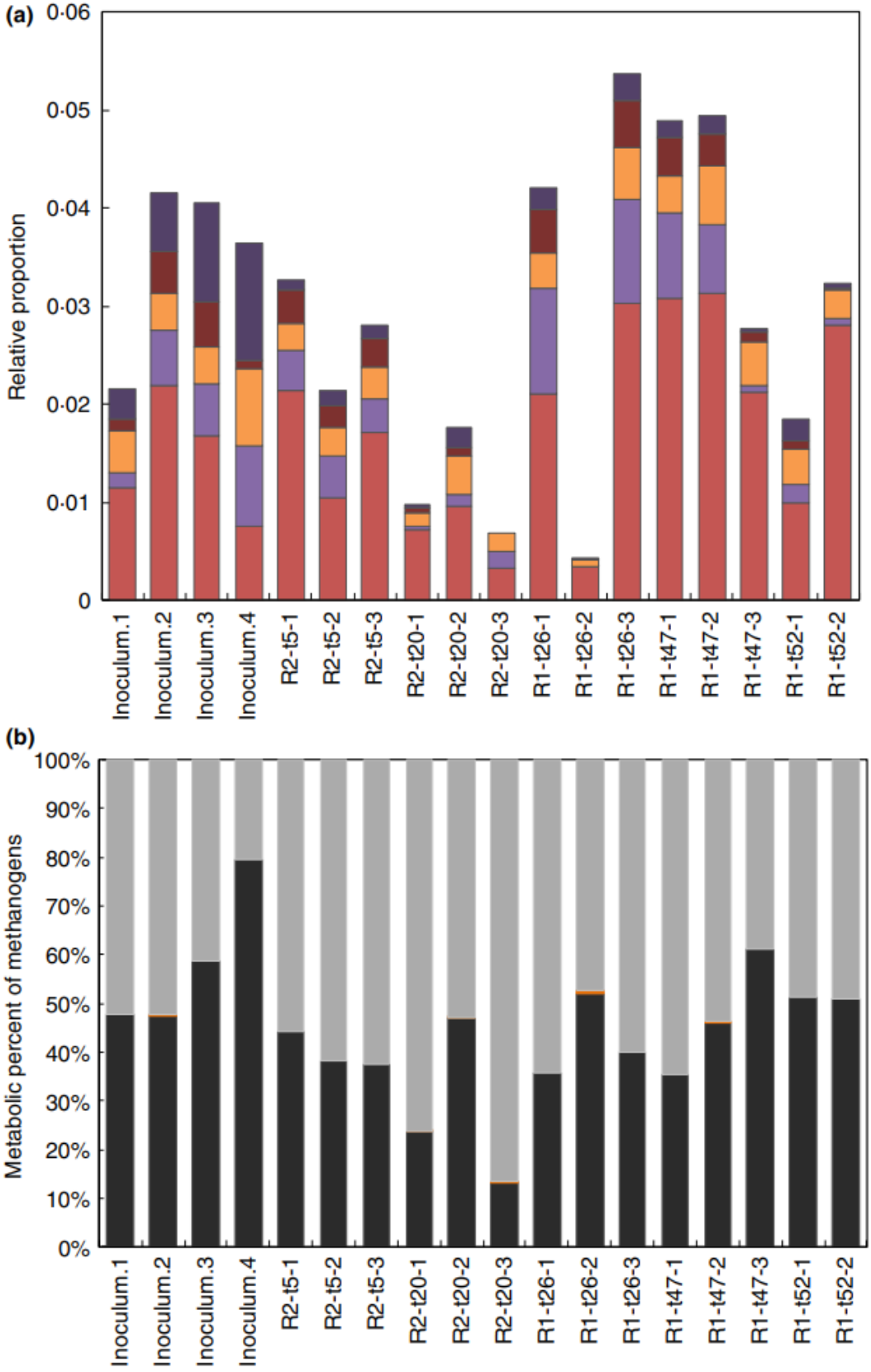
(a)
Relative proportion
0·06
0·05
0·04
0·03
0·02
0·01
0
Inoculum.1
Inoculum.2
Inoculum.3
Inoculum.4
R2-t5-1
R2-t5-2
R2-t5-3
R2-t20-1
R2-t20-2
R2-t20-3
R1-t26-1
R1-t26-2
R1-t26-3
R1-t47-1
R1-t47-2
R1-t47-3
R1-t52-1
R1-t52-2
(b)
Metabolic percent of methanogens
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Inoculum.1
Inoculum.2
Inoculum.3
Inoculum.4
R2-t5-1
R2-t5-2
R2-t5-3
R2-t20-1
R2-t20-2
R2-t20-3
R1-t26-1
R1-t26-2
R1-t26-3
R1-t47-1
R1-t47-2
R1-t47-3
R1-t52-1
R1-t52-2

Figure 3. Methanogenic archaea in continuous stirred tank reactors during settling process. (a) relative proportion of methanogenic archaea species. (■) Others; (■) Methanospirillaceae bacteria; (■) *Methanobacterium* spp. (■); *Methanolinea* spp. and (■) *Methanosaeta* spp. (b) metabolic strategies of the methanogenic archaea. (■) Hydrogenotrophic methanogens (H2); (■) Mixotrophic methanogens (Ac/H_2) and (■) Strict acetoclastic methanogens (Ac).

This study proposes the benefit of using brewery treatment plant effluents to obtain a robust consortium for methane production from anaerobic digestion of urban solid waste in Mexico, in addition to verify the changes in the community composition in response to degradation of waste water influent. The bacteria and archaea identified during the anaerobic digestion are known as relevant players that interact both in biodigestor systems and in natural communities associated to methane production. It is clear that although methanogenesis is restricted to certain groups, the stability of the consortia is fundamental to the sustained production of biogas.

Materials and methods

Substrate and inoculum obtention

A composite sample collected from organic urban residues from 24 collection points distributed in central Mexico (Mexico City, State of Mexico and Morelos), in July 2012 was used as substrate. The composition of the substrate consisted of compostable materials, with 90% associated to fruit, vegetable and gardening remains, and the remaining 10% originating from animal-based remains (dairy, meat, poultry, fish). The organic residues were collected from randomly chosen garbage trucks, fully mixed with a shovel and placed in plastic bags (c. 50 kg per site). Residues were grounded with a Nixtamatic meat grinder (Koblenz, Mexico) to reduce the particle size until 0·1–1·0 mm and finally stored at −20°C until use or analysis.

The anaerobic inoculum was a granular sludge obtained from an anaerobic brewery wastewater treatment plant in Mexico City. Granule size was determined with a laser light-scattering technique in a Mastersizer 2000 (Malvern Instruments Ltd., Malvern, UK). Granules were black and with a diameter between 0·4–1·6 mm (median, 0·8 mm). Samples were taken in sterile plastic containers and kept at 4°C until the inoculation process.

Laboratory scale reactors

Two semi-continuous stirred tank reactors (CSTR, 5 l cylindrical glass containers, 55 cm height and 14 cm diameter) were used to investigate the changes in microbial community during the start-up process. The inoculum was activated with a preincubation process in both reactors (c. 61 g l^{-1} in volatile solids (VS) basis), using a working volume of 3·5 l at 35 ± 2°C (45 rev min^{-1}) under anaerobic conditions during 5 days. After the activation process, reactors were fed the organic residues at an organic load of 4·0 kg VS m^{-3} day^{-1}, at a hydraulic retention time of 13 days, using a dilution factor of 10% total solids. The influent pH was previously adjusted to 7 with a phosphate buffer solution. Effluent and influent diary volumes were equal to maintain a constant working volume.

Total biogas production was measured by the principles of liquid displacement and buoyancy, and reported as normalized millilitres of produced biogas. The effluent was analysed for total solids (TS), volatile solids, pH and alkalinity according to standard methods. Briefly, total solids (TS) and volatile solids (VS) were determined by gravimetric methods according to Standard Methods (APHA 2540 E) (APHA 2005). pH was measured with a 6231 pH/mV/Temp METER (Jenco Instruments Inc., San Diego, CA, USA). The alkalinity was determined by alkalimetric titration (Jenkins *et al*. 1983), and the alpha index (α) was calculated as the quotient of partial alkalinity (pH 5·75) and total alkalinity (pH 4·30). Average methane content was determined in random samples with an Agilent Technology 4890D gas chromatograph fitted with a flame ionization detector (FID). Further details of the methodology can be found in Ruíz-Valdiviezo *et al*. (2010).

DNA extraction from reactor biomass and PCR amplification

The composition of the microbial consortia in each reactor was analysed from samples of the granular sludge during different times of operation. Samples of reactor biomass were taken after 0, 5, 20, 26, 47 and 52 days of operation (R1-t0, R1-t26, R1-t47, R1-t52, R2-t0, R2-t5, R2-t20). For each sampling time, three sludge subsamples (c. 15 g) were taken in sterile Falcon tubes, and kept at −20°C until processing. DNA was extracted from 300 mg of biomass per subsample using the modified protocol reported from Centeno *et al*. (2012). In this case, the originally proposed freeze-thaw cycles were avoided and samples were disrupted with a bead-beater (FastPrep FP120 Homogenizer, ThermoSavant, Carlsbad, CA, USA). DNA pellets were washed twice with ethanol 80%, dried at 50°C (15 min) and eluted with 40 μl molecular grade water (Sigma Aldrich, Saint Louis, MO, USA). Samples were kept at −20°C until processing. Paired-end 16S rRNA gene community sequencing was done using primers universal primers for archaea and bacteria 515F/806R (region V4) (Caporaso *et al*. 2010, 2012). Subsamples were treated as separate samples, and each PCR reaction contained a specific Golay reverse primer (Caporaso *et al*. 2010). PCR reactions contained 2 ng μl^{-1} of template, 2·5 μl Takara ExTaq PCR 10× buffer (TaKaRa Corp., Shiga, Japan), 2 μl Takara dNTP mix (2·5 mmol l^{-1}), 0·7 μl bovine serum albumin (20 mg ml^{-1}, Roche, Mannheim, Germany), forward and reverse primers (10 μmol l^{-1} final concentration), 0·625 U Takara Ex Taq DNA Polymerase and nuclease free-water until 25 μl. Amplification protocol included a denaturalization step at 95°C for 3 min, followed by 35 cycles of 95°C, 30 s, 52°C, 40 s, 72°C, 90 s, and a final extension at 72°C for 12 min.

DNA sequencing and analysis

Amplicons (~20 ng per sample) were sequenced on an Illumina MiSeq platform (at the Yale Center for Genome Analysis, CT), resulting in ~250 bp paired-end reads. The paired-end sequences were overlapped and merged using FLASH (Magoč and Salzberg 2011). Quality filtering and demultiplexing

were performed as previously reported (Caporaso *et al*. 2012; Bokulich *et al*. 2013). Chimeric sequences were detected and removed with USEARCH (Edgar 2010), while singletons were not considered for analysis. Taxonomic assignments were done with QIIME (Caporaso *et al*. 2010) ver. 1.7.0 using the RDP classifier (Wang *et al*. 2007) and the Greengenes database (release 13_5). Samples were rarefied to 2000 sequences. The sequence data are available from the NCBI BioProject No. SUB1384244.

A constrained or “canonical” correspondence analysis (CCA) was performed to visualize differences between the community composition (at class level) and CSTRs physicochemical environment. The representation in a Euclidean space was obtained with the function ‘cca’ in ‘vegan’, based on Legendre and Legendre's (2012) algorithm. The graphic was done with ‘ggvegan’. All statistics packages were used in the R statistical environment ver. 3.3.2 (R Development Core Team 2012).

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