

## Substrate influence on the structure of methanogenic Archaea communities during anaerobic digestion

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### ABSTRACT

This study compares the diversity of methanogenic archaeal communities that developed during biogas production in reactors fed with different substrates. Reactor I was fed with silages of maize and of alfalfa and timothy; and Reactor II was fed with these silages plus pig slurry and glycerol as co-substrates. The archaeal community structure was studied using polymerase chain reaction–denaturing gradient gel

electrophoresis based on the *16S rRNA* gene. In both reactors, *Methanosphaerula palustris* was most abundant, and species belonging to *Methanolinea*, *Methanoculleus*, and *Methanotorris* were present. Only Reactor I, where the ammonium concentration was lower, had species belonging to *Methanospirillum* and *Methanosarcina*. Thus, it appears that addition of pig slurry increased the ammonium concentration, which inhibited the growth of *Methanospirillum* and *Methanosarcina*.

### INTRODUCTION

Methane fermentation has recently been a subject of much interest because methane is a renewable energy source and fermentation provides a way to utilize waste. For better methane production, the process parameters can be adjusted for the specific waste product being used as a substrate, and co-substrates can be added. In this way, greater process stability can be achieved, and the quantity and methane content of biogas can be increased (Bułkowska et al. 2012).

Although it is known that the Archaea are one of the groups of microorganisms that perform methane fermentation, their community structure and diversity are poorly understood, as is the effect of conditions in the

bioreactor on these community characteristics (Ciesielski et al. 2013). For example, the choice of feedstock can affect these characteristics (Ziganshin et al. 2013). The feedstock that is chosen can come from a variety of industries, and its choice can depend on the availability of raw materials. Although in Europe, the materials most commonly used in biogas production are plant-based, some animal-derived organic wastes and other organic wastes are also used, such as pig slurry or glycerol from the biodiesel industry (Hijazi et al. 2016). The effect on biogas production of addition of substrates that are not plant-based has been investigated (Bułkowska et al. 2012). However, little is known about how the diversity of the archaeal community is affected when glycerol and pig slurry are added as co-substrates to plant-based substrates.

Therefore, the present study compared the diversity in the methanogenic archaeal community when using only silages as substrates, and using silages plus glycerol and pig slurry. The *16S rRNA* gene was analyzed with DGGE and the Sanger method of DNA sequencing. Here, we report that the addition of pig slurry and glycerol decreased the variety of Archaea species.

## MATERIALS AND METHODS

### Materials

The material used in the study was waste digestate from two anaerobic Continuous Stirred-Tank Reactors (CSTR). Reactor I was fed with a mixture of two silages: silage made of maize, and silage made of alfalfa and timothy at a ratio of 90:10%. Reactor II was fed with the mixture of silages plus co-substrates: silage made of maize, and silage made of alfalfa and timothy, plus pig slurry and glycerol at ratios of 87.5:6.26:6.25%. The experiments were conducted with a working volume of 6L.

Samples were taken from Reactor I on days 32, 37, 41, 45, 54, 58, and 61 of the process, and from Reactor II on days 18, 23, 31, 37, 38, 42, 44, and 47. Both digesters were operated at a hydraulic retention time (HRT) of 45 days, an organic load rate of  $2.0\text{g}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$ , and a temperature of  $39^\circ\text{C}$ .

During digestion, the pH and chemical oxygen demand (COD) of the digestate were determined, and the volume of biogas, and the contents of methane, ammonium, and volatile fatty acids (VFAs) were measured. The pH was measured immediately after sampling. COD, ammonium nitrogen and VFAs were measured in filtered supernatant centrifuged for 10 minutes at  $8693\text{g}$  (APHA 1992). Biogas was collected in Tedlar sample bags for 24h and its volume was measured using a gas meter. The content of methane, carbon dioxide and oxygen in the biogas was determined using a GA 2000+ automatic analyser (Geotechnic Instruments, UK).

### Molecular analysis of the Archaea community structure

#### DNA extraction

75mg of biomass were weighed in an Eppendorf tube. Next, 150mg of glass beads with a diameter of 1.25-1.55mm and 150mg of beads with a diameter of 0.4-0.6mm were added, along with 1mL of extraction buffer (100mM Tris-HCl, 100mM EDTA, 1.5M NaCl, pH=8). The samples were shaken in a bead-beating device for 20 minutes at 5000rpm. Then 200mL of 10% SDS solution were added before incubation for 30 minutes at  $65^\circ\text{C}$  in a Thermomixer. After incubation, the samples were centrifuged for 10 minutes at 8000rpm and 0.5mL of supernatant was transferred to a column for DNA purification (A&A Biotechnology, according to the manufacturer's recommendations). The

column was then centrifuged for 1 minute at high speed before the filtrate was poured out and washed twice with A1 solution (A&A Biotechnology). The DNA was then suspended in  $50\mu\text{L}$  of water and stored at  $-20^\circ\text{C}$  until further analysis.

#### Polymerase chain reaction

Genomic DNA was amplified using polymerase chain reaction (PCR). The gene fragment encoding for 16S rRNA was amplified using a pair of primers (GC-0357F-5' CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCC CGCCCGCCCTACGGGGCGCAGCAG 3'; 0691R-5' GG ATTACATGATTTTCAC 3') (Watanabe et al. 2004). The amplified fragments measured approximately 500bp.

The PCR mix ( $30\mu\text{L}$  per reaction) was composed of  $3\mu\text{L}$  of  $10\times$ PCR buffer,  $2.4\mu\text{L}$   $\text{MgCl}_2$  (25mM),  $1.3\mu\text{L}$  of dNTPs ( $200\mu\text{M}$  final concentration),  $0.15\mu\text{L}$  Taq polymerase ( $2\text{U}\cdot\text{L}^{-1}\cdot\text{reaction}^{-1}$ ),  $0.5\mu\text{L}$  of each primer (20pmol),  $18.15\mu\text{L}$  of  $\text{dH}_2\text{O}$  and  $1\mu\text{L}$  of genomic DNA. Reactions were performed in 0.5mL DNA-free PCR tubes using a thermocycler, and the PCR steps were as follows: denaturation at  $94^\circ\text{C}$  for 10min, followed by 30 cycles of denaturation at  $94^\circ\text{C}$  for 1min, annealing at  $54^\circ\text{C}$  for 1min in the initial cycle, and then for a period that was 2 seconds shorter after each subsequent cycle, and extension at  $72^\circ\text{C}$  for 1min. After completion, an additional extension step was performed at  $72^\circ\text{C}$  for 10min, and the samples were then chilled to  $4^\circ\text{C}$ . The length of the PCR product was verified on 1% agarose gel, stained with ethidium bromide, and visualized and photographed under UV light.

#### Denaturing Gradient Gel Electrophoresis (DGGE)

PCR products with a GC clamp were resolved in 6% polyacrylamide gel (37.5:1 acrylamide:bisacrylamide) with a gradient ranging from 30 to 60% urea. Electrophoresis was performed for 12h at 60V in 1xTAE buffer (2M Tris base, 2M acetic acid, 0.05M EDTA) using the DCode™ Universal Mutation Detection System (Bio-Rad Laboratories Inc., U.S.A.). The DNA mixture resolved in gel was visualized by staining with 1:10,000 SybrGold (Invitrogen) for 20 minutes followed by UV trans-illumination. Images were recorded and analyzed with KODAK 1D 3.6 Image Analysis Software.

#### DNA sequencing

The *16S rRNA* gene bands with the greatest intensity were excised from the denaturing gel, transferred into  $50\mu\text{L}$  sterile water and frozen at  $-20^\circ\text{C}$  for 24h. The samples were then thawed at room temperature, and gel fragments were shredded using a glass rod. Eluted PCR products were then re-amplified using the same set of primers. PCR products were purified using a Clean-up kit (A&A Biotechnology, Poland) and cloned using an InsTAclone PCR Cloning Kit

(Thermo Scientific). Plasmid DNA was purified using a Plasmid DNA Kit (A&A Biotechnology, Poland). Sequencing reactions were carried out with ABI3730XL (PE Applied Biosystems). All reactions were run following the manufacturers' protocols. The nucleotide sequences were submitted to the GenBank database under accession numbers from KM437893 to KM437899.

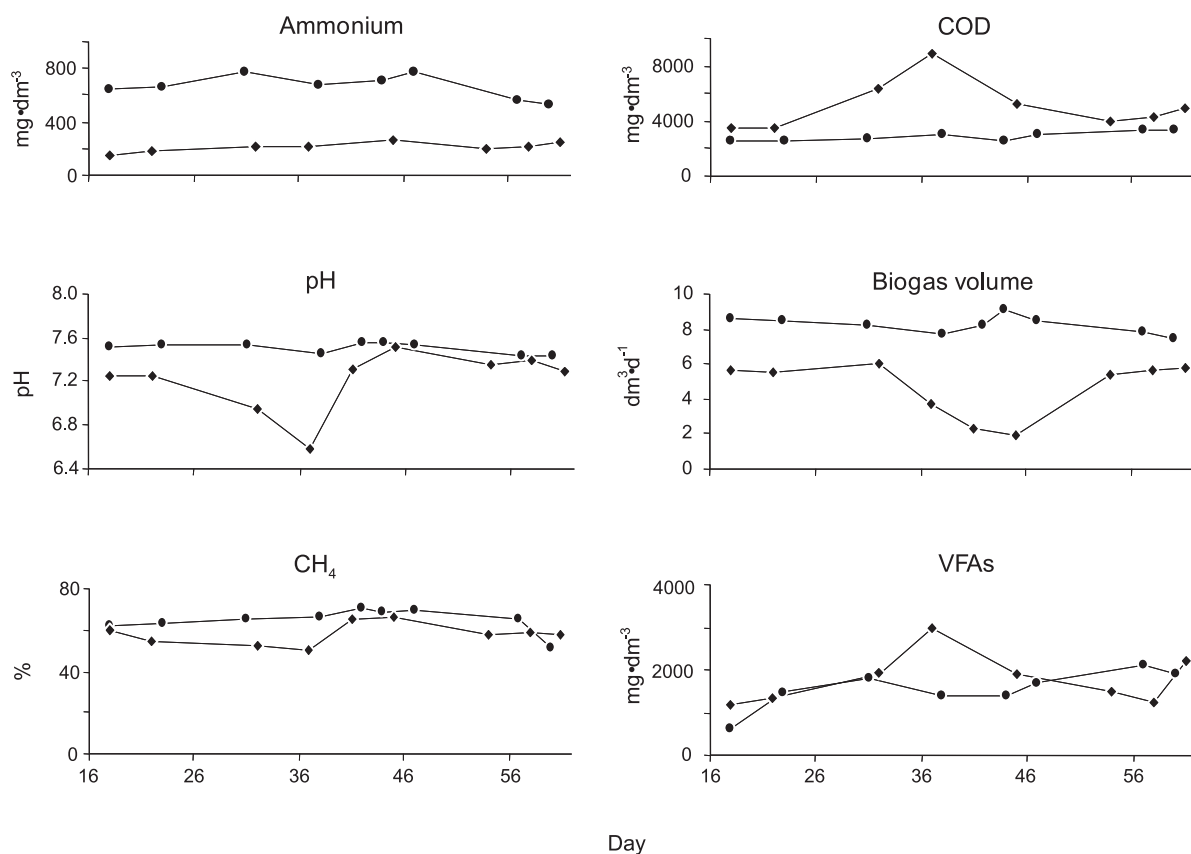
### Phylogenetic analysis

The sequences obtained for *16S rRNA* genes were compared with those from the GenBank database using the NCBI Blast program. The sequences were aligned using the ClustalW program (Thompson et al. 1994). Genetic relationships were determined by the neighbor-joining method with the MEGA2 program (Kumar et al. 2001) using nucleotide sequences of the *16S rRNA* gene. To infer the consensus tree, 1,000 bootstrap replicates of data were analyzed.

### Statistical analysis

The DGGE patterns were converted to a binary matrix, using presence-absence data. The pairwise similarity of the banding patterns of the different samples was calculated applying Nei-Li distance (Nei and Li 1979) and an UPGMA cluster analysis was conducted using DGGEstat software (Erik van Hannen, the Netherlands Institute of Ecology). To determine if the differences in community structure were statistically significant, Nei-Li distances and Student's t-test were used. Two groups were considered to differ significantly if the Nei-Li distance between them was significantly greater than the Nei-Li distance between samples that were taken from the same group (van Hannen et al. 1999).

## RESULTS



**Figure 1.** Physico-chemical characteristics of the methane fermentation processes. Diamond lines show Reactor I (fed with silages of maize and alfalfa alone), while circle lines show Reactor II (fed with silages plus pig slurry and glycerol as co-substrates).

From days 16 to 36, Reactor I (with maize and alfalfa silages alone) had a higher COD and concentration of VFAs and a lower concentration of ammonia than Reactor II (with the

same silages plus pig slurry and glycerol as co-substrates). Reactor I also had a lower pH, and produced a smaller volume of biogas with a smaller percentage of methane (Figure 1).

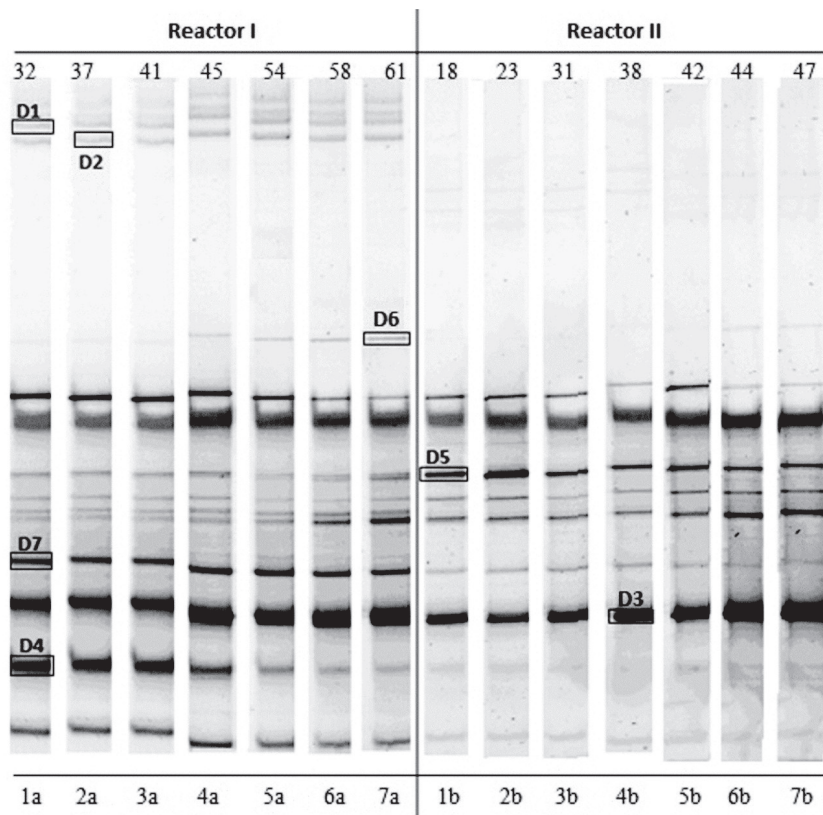


Figure 2. DGGE profiles of bacterial 16S rDNA fragments amplified from genomic DNA extracted from samples coming from anaerobic Reactor I, fed with silages of maize and alfalfa alone (lanes 1a-7a) and from Reactor II, fed with these silages plus pig slurry and glycerol as co-substrates (lanes 1b-7b). Numbers on the top indicate days of anaerobic fermentation, D1-D7 show the DNA bands which were excised from the gel and sequenced.

*16S rRNA* gene analysis with DGGE showed a number of bands that appeared in both reactors throughout the process. These bands were in the bottom part of the gel, and the intensity of these bands differed between the reactors (Figure 2). In Reactor I, the intensity of band D5 varied, but was never as intense as this band in Reactor II. Bands D4 and D7, in contrast, were more intense in Reactor I than in Reactor II.

A number of bands appeared only in Reactor I: bands D1, D2 and D6. These results indicate that the Archaeal community was more diverse in Reactor I than in Reactor II. The tree of genetic distances between the Archaea showed distinct clades. Samples from Reactor I were more homogenous than those coming from Reactor II. Genetic distance was significantly greater between reactors than between the first three samples and the last four samples from Reactor II (Student's t-test,  $p < 0.05$ ) (Figure 3).

The BLAST program showed similarities between the analyzed DNA sequences and those deposited in

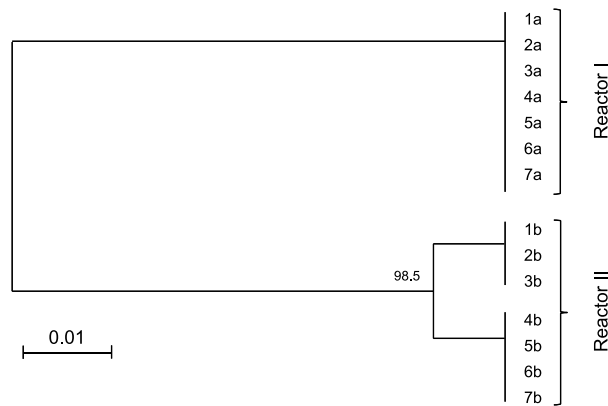


Figure 3. Dendrogram showing cluster analysis of the digital profiles from Figure 2, using the unweighted pairwise grouping method with mathematical averages (UPGMA) (Dice coefficient of similarity). The analysis takes into account the presence or absence of bands at certain positions in each lane. The number at the branching point is the value of 1,000 bootstrap replicates. The scale bar indicates the distance between clusters in UniFrac units.

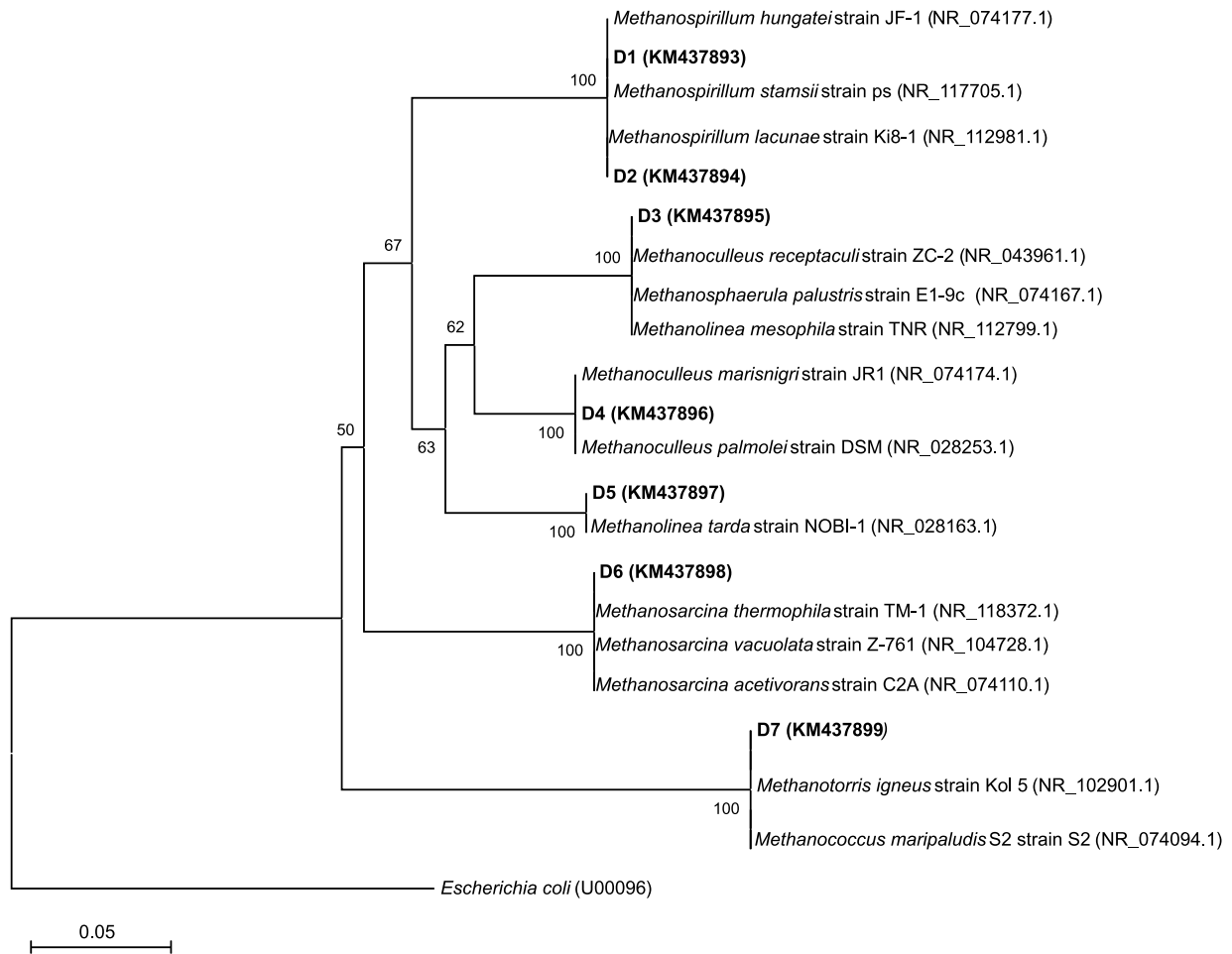


Figure 4. Phylogenetic analysis of representative *16S rRNA* gene nucleotide sequences from the NCBI sequence database. The tree was calculated by a nucleotide alignment of *16S rRNA* gene fragments using the neighbour-joining method. *Escherichia coli* (accession U00096) was used as the outgroup. The scale bar represents 5% *16S rRNA* nucleotide sequence divergence per homologous position. Phylogenetic analysis grouped all of the analyzed DNA sequences into three orders: *Methanococcales*, *Methanosarcinales*, *Methanomicrobiales*.

Table 1. Similarity between Archaeal sequences obtained in this study and those previously deposited in GenBank.

DNA band (accession number)	Previously deposited DNA sequence (taxon name and accession number)	Similarity (%)	Metabolic group
D1 (KM437893)	<i>Methanospirillum hungatei</i> JF-1 (NR_074177)	99	hydrogenotrophic
D2 (KM437894)	<i>Methanospirillum hungatei</i> JF-1 (NR_074177)	99	hydrogenotrophic
D3 (KM437895)	<i>Methanosphaerula palustris</i> E1-9c (NR_074167)	92	hydrogenotrophic
D4 (KM437896)	<i>Methanoculleus receptaculi</i> ZC-2 (NR_043961)	99	hydrogenotrophic
D5 (KM437897)	<i>Methanolinea mesophila</i> TNR (NR_112799)	97	hydrogenotrophic
D6 (KM437898)	<i>Methanosarcina thermophila</i> TM-1 (NR_118372)	99	acetotrophic
D7 (KM437899)	<i>Methanotorris formicicus</i> Mc-S-70 (NR_028646)	84	hydrogenotrophic

GenBank (Table 1). Phylogenetic analysis grouped the analyzed DNA sequences into several clusters (Figure 4), representing three different orders: *Methanomicrobiales* (bands D1-D5), *Methanosarcinales* (band D6), and *Methanococcales* (band D7). The only acetotrophic methanogen identified in this study was *Methanosarcina thermophila* (band D6), which was found only in Reactor I (Figure 2). All the other bands corresponded to hydrogenotrophic methanogens.

## DISCUSSION

The present study compares the diversity in the methanogenic archaeal communities that developed during biogas production in Reactor I (with maize and alfalfa silages alone) and Reactor II (with these silages plus pig slurry and glycerol as co-substrates). *16S rRNA* gene analysis with DGGE and the Sanger method of DNA sequencing revealed a number of bands, among which bands D1–D7 were most intense. These bands were identified as representing Archaea which belong to the group using  $H_2/CO_2$  as a substrate.

The most intense band in both reactors was D3. Its DNA sequence was similar to *Methanosphaerula palustris*, which uses formate and  $H_2/CO_2$  as a substrate (Cadillo Quiroz et al. 2009). The occurrence of this species was also reported by Kim et al. (2013), who conducted methane fermentation while treating swine wastewater at laboratory scale and in a pilot plant.

Bands corresponding to *Methanoculleus receptaculii* (D4) and *Methanotorris formicicus* (D7) were most intense in Reactor I, which resulted from the acidic environment in this reactor. Both taxa are hydrogenotrophic methanogens which use  $H_2/CO_2$ . Although methane fermentation typically takes place at pH 7.0–8.0, the pH in Reactor I was substantially lower up to the 46<sup>th</sup> day of the process. Under such conditions, only hydrogenotrophic methanogenesis is possible because the microorganisms that perform this process can tolerate the acidic environment (Kim et al. 2004). *Methanoculleus* sp. was first isolated from an anaerobic, propionate-degradation enrichment culture that was originally established from a rice field soil sample from Taiwan (Sakai et al. 2012). Although several studies have found that *Methanoculleus* sp. was the dominant microorganism in the reactor (Klocke et al. 2008; Krause et al. 2008; Kröber et al. 2009), to our knowledge this is the first report of this microorganism in anaerobic conditions with slurry and glycerol additives.

Band D5, corresponding to *Methanolinea mesophila*, was also observed in both reactors. In Reactor I the intensity of band D5 varied, but it was never as intense as in Reactor II, where the concentration of ammonium was very high.

Bands D1, D2, and D6 were only in Reactor I, indicating that the archaeal community in this reactor was more diverse than in Reactor II. Bands D1 and D2 corresponded to *Methanospirillum hungatei*, which uses formate to produce

methane because it is unable to consume acetate and ethanol (Crabble et al. 2011). Band D6, corresponding to *Methanosarcina thermophila* TM-1, was present only in Reactor I. This taxon often occurs in microbial communities producing methane from plant substrates, as was the case in this reactor. This taxon can form multicellular aggregates, which may help it to resist inhibition by VFAs. This is because, inside the aggregates, the concentration of VFAs is reduced, due to the low rate at which these acids diffuse into the aggregates (Vavilin et al. 2008). Whether these aggregates helped *Methanosarcina thermophila* to resist inhibition or not, the abundance of this taxon in Reactor I increased when the concentrations of VFAs were lower in the second part of the process (Figure 1). It is also probable that these methanogens are sensitive to higher concentrations of ammonium (Yenigün and Demirel 2013), which is why this species did not occur in Reactor II.

## SUMMARY AND CONCLUSION

*16S rRNA* gene analysis with DGGE and the Sanger method of DNA sequencing showed that most of the detected Archaea were hydrogenotrophs. The archaeal community was more diverse in Reactor I (fed with maize and alfalfa silage alone) than in Reactor II (fed with the same silages plus glycerol and pig slurry as co-substrates). As glycerol is an excellent substrate for the growth of most microorganisms, it can be concluded that the addition of pig slurry, possibly due to its high ammonium concentration, inhibited the growth of *Methanosarcina*, *Methanoculleus* and *Methanospirillum*.

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