Microbial community response to transitional states in anaerobic digesters

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Abstract
A better understanding of the microbial ecology of anaerobic processes during transitional states is important to achieve a long-term efficient reactor operation. Five wastes (pig manure, biodiesel residues, ethanol stillage, molasses residues and canning waste) were treated in five anaerobic reactors at the same operational conditions. The influence of the type of substrate and the effect of modifying the feeding composition on the microbial ecology was evaluated. The highest biomethanation percentages were observed in canning waste reactors which also presented the highest archaeal percentages. Only two *Bacteria* populations could be related with the substrate, *Ilyobacter* with biodiesel residues and *Trichococcus* with molasses residue. Results showed that the length of the start-up period or the adaptation time after a change in the feeding composition is not dependent on the type of substrate. However, the cluster analysis showed that substrate had a clear influence in the microbiology of the reactors. Reactors with better biomethanation yields tended to have more diverse microbial communities. The lowest the microbial community organization value (more even communities) the poorest the reactor performance. It implies that highly even communities are not a clear evidence of better operation.

Keywords
Agro-industrial wastes; community organization; Denaturing Gradient Gel Electrophoresis (DGGE); *Ilyobacter;* Microbial Resource Management (MRM).

INTRODUCTION

Several authors have followed the microbial population dynamics during different stages of the anaerobic digestion process and most of them have been focused on steady state processes. Dearman et al. (2006) showed that a great microbial diversity is not necessary to operate successfully anaerobic reactors. Carballa et al. (2011) found that the populations under mesophilic conditions were more diverse and balanced compared to the thermophilic ones. Moreover, the latter study showed that a high rate of change of the microbial community composition and initial community evenness in the start-up are important factors for keeping stable operation of mixed microbial cultures. Werner et al. (2011) highlighted the importance of syntrophic populations in anaerobic reactors, underlining their resilience. However, few data is available on microbial community's behaviour under transitional stages, such as start-up or changes in the feeding composition. This information is necessary to predict and prevent eventual instability problems in such critical steps.

The objective of this study was to monitor and evaluate microbial community dynamics in five anaerobic reactors fed with five different agro-industrial wastes in transitional states. The influence of substrate characteristics on the anaerobic community structure and the response of the microbial community structure against the changes in the feeding composition were analyzed.

METHODS

Substrates

Five agro-industrial wastes were chosen according to their different physico-chemical characteristics: pig manure (PM), biodiesel residues (BR), ethanol stillage (ES), molasses residues (MR) and canning waste (CW). The main characteristics of each substrate are presented in Table 1.

Anaerobic reactors

Five mesophilic continuously stirred tank reactors with a working volume of 2 L were used. The digesters were inoculated with a mixture of seven anaerobic sludges to obtain the greatest possible initial microbial diversity. All the reactors were operated at the same organic load rate (OLR) of 1 g chemical oxygen demand (COD) L^{-1} d^{-1}) and at a hydraulic retencion time of 20 days during the whole experiment (210 days). Two different stages could be distinguished: stage 1, treating a different substrate in each reactor, lasted 115 days, and stage 2, when the substrates were exchanged, lasted 95 days. Table 2 shows the substrates fed in the first and second stages in each reactor.

The biogas production was measured daily by liquid displacement and the pH was monitored three times per week. Biogas composition was analysed by gas chromatography (HP, 5890 Series II). Samples from the supernatant of the reactors were taken three times a week for COD, $N\text{-}NH_4^+$ and volatile fatty acids (VFA) determinations. Once a week, a sample of the anaerobic biomass was taken from each reactor for molecular analyses. The biomass samples for Fluorescent in situ hybridization (FISH) were fixed the same day of sampling, while samples for Denaturing Gradient Gel Electrophoresis (DGGE) were stored at -20 °C until DNA extraction. DGGE plus sequencing and FISH analyses were performed according to Regueiro et al. (2012).

Table 1. Substrates characteristics (in g L^{-1}).

Parameter	PM	BR	ES	MR	CW
TS	20	593	438	835	304
VS	10	557	422	707	282
COD_{Total}	14	1679	374	723	567
$N\text{-}NH_4^+$	2.2	0	0.4	14.8	0.7
Lipids	0.0	2.2	0.0	0.0	35.1

TS: total solids; VS: volatile solids; COD: chemical oxygen demand.

Table 2. Exchange of substrates

	Stage	
Reactor	1	2
1	PM	CW
2	BR	CW
3	ES	MR
4	MR	CW
5	CW	PM

Statistical analysis

Analysis of the obtained DGGE patterns was done with BioNumerics software v.6.1 (Applied Maths, Sint-Martens-Latem, Belgium). Profile similarities were obtained by determination of the Jaccard coefficient. Cluster analyses were constructed using UPGMA algorithm. The range-weighted richness (Rr), the community organization (Co) and also the dynamics (Dy) were calculated according to Marzorati et al. (2008).

RESULTS AND DISCUSSION

Stage 1

Figure 1A shows a stable performance in all the digesters after approximately 30 days. Among the different wastes, the highest organic matter removal efficiencies (Figure 1B) were observed in the reactors treating BR, MR and CW (70-90%). The other two reactors, operating with PM and ES, showed poorer results (45-55%), probably due to the high amount of slowly biodegradable organic matter. The percentage of methane in the biogas was similar in all the reactors (45-55%).

DGGE results revealed that there was not a clear relationship between the species and the substrate fed, since in all the reactors the most abundant bacterial populations belong to *Firmicutes* and *Proteobacteria* phyla and among the archaeal communities *Methanolinea*, *Arc1* and *Methanosarcinales* were dominant. Yet, two species only appeared in two reactors, *Ilyobacter* in the BR reactor and *Trichococcus* in the MR one.

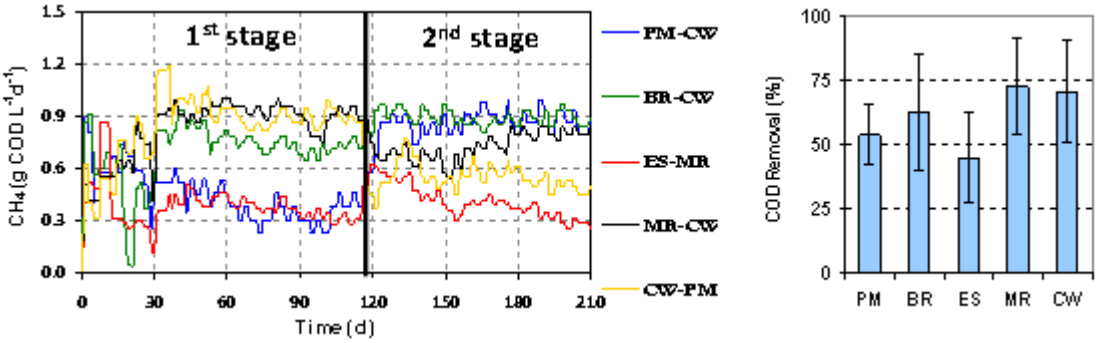

Figure 1. Volumetric methane production (A) and average COD removal efficiencies during the first stage (B) in the 5 reactors.

The community structure changed over the time as a result of the biomass adaptation to each specific substrate. Cluster analysis of *Bacteria* population showed a segregation of samples according to the sampling time (Figure 2A). *Bacteria* population needed almost 60 days to achieve the microbial steady state conditions in all the reactors studied, except for the CW reactor, in which only needed 44 days. *Archaea* clusters (data not shown) followed the same chronological pattern, but this population reached microbial steady state in only 20-30 days, in accordance with the steady state in physico-chemical parameters and biogas production. According to these results it can be said that the type of substrate did not affect the duration of the start-up period, since the five reactors achieved steady state conditions at the same time. These results agree with those presented by Liu et al. (2002) where less than 2 weeks are needed to establish a desire microbial community in acidogenic reactors.

The influence of the substrate fed on the microbial population structure was analyzed with the cluster results. Figure 2B shows the cluster analysis for the *Bacteria* population at the end of stage 1(day 110). Two different groups can be observed: PM and ES, similar to each other by 30%, and CW and BR similar to each other by 40%. Reactor treating MR differed from the others the most, probably due to the high ammonium concentration detected in this reactor (≈4 g $N\text{-}NH_4^+$ L^{-1}). *Archaea* population presented similar cluster results. Therefore the type of substrate affected the population structure of anaerobic digesters since all reactors started with the same inoculum. Moreover, the structure is also affected by the operational parameters, such as COD removal (Figure 1B) and ammonium concentration.

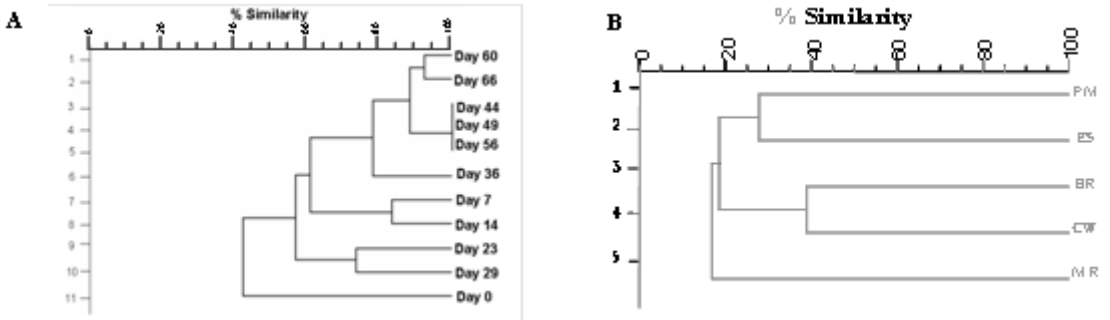

Figure 2. Cluster analysis of *Bacteria* during the first experimental part for the CW reactor (A), Cluster analysis of *Bacteria* at the end of the first stage for the 5 reactors (B).

Rr values were similar for both populations (10-45 for *Bacteria* and 5-50 for *Archaea*) and decreased over time. The highest values were obtained for the BR and MR reactors. They also had the better biomethanation yields, indicating that the more diverse microbial communities the better the reactor performance. Co remained constant over the time in each reactor, with average values ranging from 35 to 60 which were representative of balanced microbial communities and functional stability (Werner et al., 2011). Bacterial communities tended to have higher average Co values indicating more unevenness than the archaeal ones. These results were in the same range as those

found in literature (Carballa et al., 2011). The lowest Co values (15-20) were observed in the reactor with the poorest performance (ES), suggesting that even communities (lower Co values) might not be indicative of a well-functioning reactor. Moreover, all the reactors showed high Dy values during the first weeks (40-70% rate of change per week), decreasing later to almost negligible values (0-10%). The archaeal communities were fairly less dynamics compared with the bacterial ones. FISH analyses indicated that the ratio *Archaea*/*Bacteria* decreased over time and that the higher the archaeal percentage (BR and CW reactors) the better the reactor performance. *Methanosaeta* and *Firmicutes* were the dominant specie and phylum in the *Archaea* and *Bacteria* population, respectively. *Methanosarcina* appeared in CW reactor probably due to high salt concentrations.

Stage 2

Figure 1A also shows a stable performance in only 10-12 days in this stage. DGGE showed that the most abundant bacterial populations were *Firmicutes* and *Clostridium*, in MR and PM reactors, respectively. *Trichococcus* (belong to *Firmicutes*) appeared again in MR reactor. Microbial steady-state was achieved earlier than in stage 1 (20 days for Bacterial population and 10 for the Archaeal one). This fact indicates that the start-up is more critical than a change in the substrate fed to reach stable performance. Also it shows that recovery time after a change in feeding composition is not dependent on the type of substrate. Rr values for *Archaea* were lower (0-10) than *Bacteria* (5-40). In this stage the Rr values were slightly lower compared to the first one. Again, the lowest Co values (10-15) were observed in the reactor with the poorest performance (MR). The Dy values decreased over time but reached negligible values in only few days (7-15 days). FISH results also showed that the archaeal percentage was higher in the CW reactor (50%).

CONCLUSIONS

This study demonstrates that the start-up duration or the adaptation period after a change in the feeding is not related to the type of substrate. However the substrate clearly determines the microbial community structure of the anaerobic reactors. Two *Bacteria* populations, *Ilyobacter* and *Trichococcus* could be related with the substrate, biodiesel and molasses residues, respectively. Furthermore the high archaeal abundance and the high diversity of the microbial communities are closely related to the biomethanation efficiency.

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