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Analysis of methanogenic activity in a thermophilic-dry anaerobic reactor: Use of fluorescent *in situ* hybridization

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ABSTRACT

Methanogenic activity in a thermophilic-dry anaerobic reactor was determined by comparing the amount of methane generated for each of the organic loading rates with the size of the total and specific methanogenic population, as determined by fluorescent *in situ* hybridization. A high correlation was evident between the total methanogenic activity and retention time [$-0.6988\mathrm{Ln}(x) + 2.667$] ($R^2$ 0.8866). The total methanogenic activity increased from 0.04×10^{-8} $mLCH_4$ $cell^{-1}day^{-1}$ to 0.38×10^{-8} $mLCH_4$ $cell^{-1}day^{-1}$ while the retention time decreased, augmenting the organic loading rates. The specific methanogenic activities of H_2-utilizing methanogens and acetate-utilizing methanogens increased until they stabilised at 0.64×10^{-8} $mLCH_4$ $cell^{-1}day^{-1}$ and 0.33×10^{-8} $mLCH_4$ $cell^{-1}day^{-1}$, respectively. The methanogenic activity of H_2-utilizing methanogens was higher than acetate-utilizing methanogens, indicating that maintaining a low partial pressure of hydrogen does not inhibit the acetoclastic methanogenesis or the anaerobic process.

1. Introduction

Anaerobic methanogenic fermentation is a multi-stage process catalyzed by the coordinated activity of a wide number of individual microbial strains belonging to a number of different trophic groups. Methanogenic populations catalyze the terminal stage of the process and are normally divided into two main groups, based on their substrate conversion capabilities. Acetoclastic methanogens are capable of converting acetate to methane and CO_2, and are regarded as playing a dominant role in CH_4 production since around 70% of methane produced in digesters comes from acetate (Zinder, 1993). Hydrogenotrophic methanogens convert H_2/CO_2 to methane. These species also play a key role in the overall process by maintaining a low partial pressure of H_2 (<10 Pa). This is necessary for the functioning of the intermediate trophic group, the syntrophic bacteria, which are responsible for the conversion of organic acid and alcohol intermediates to direct methane precursors (McInerney et al., 1980; Pauss et al., 1990).

From the standpoint of the design and operation of anaerobic processes, methanogenic 'activity' is of great importance. Therefore, it is important to recognize that the amount of active methanogenic population in an anaerobic reactor is the critical factor in achieving efficient wastewater treatment. The treatment capacity of an anaerobic digestion system is influenced by wastewater composition, system configuration and operation of anaerobic digestion.

Acidogenic and methanogenic microorganisms differ, not only in terms of their nutrition and pH requirements, but also with respect to their physiology, growth, and nutrient uptake kinetics, and in their particular ability to withstand environmental changes. Methanogens are among the most fastidious of the anaerobes; they require, or are stimulated by, growth factors such as vitamins, unusual trace minerals (such as Co and Ni), fatty acids (acetate), or specific co-factors (coenzyme M) unique to methanogenic microorganisms. Consequently, conditions that are favourable to the growth of acid-forming bacteria (short HRT (1.7 days), low pH (5.2–6.5)) may be inhibitory to methane-forming microorganisms (Ince et al., 1997). Methanogenic microorganisms grow more slowly than acidogenic bacteria, at a rate similar to acetogens (3.6 days). The optimum pH environment for methanogens is within the range 6.5–8.5 (Chynoweth and Isaacson, 1987; Hobson and Wheatley, 1993; Vavilin et al., 1998).

The stability of the system depends on the active bacterial groups involved in the process. Understanding the functioning of anaerobic reactors requires quantitative information on microbial numbers, biomass, and activities of the bacterial groups involved in the process. Numbers, biomass, and activities represent distinct ecological parameters. Though normally correlated to each other, these parameters should not be used in an interchangeable manner. On occasion, when one parameter (such as number of microorganisms) is required, technical difficulties make it necessary to

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measure a less relevant parameter (such as biomass), and then to calculate the required parameter from this. For example, the lack of methods for determining the concentration and activity of the different bacterial groups involved in the anaerobic process is a significant limitation in studies on the kinetics of the process, development, operation, and monitoring of particular reactors, since the measurement of biomass (as total volatile solids, principally) does not provide this information.

Direct count procedures by microscopic methods yield the highest estimates of numbers of microorganisms and are occasionally used for indirect calculation of biomass. Epifluorescence microscopy with fluorometric stains is widely used for direct counting of bacteria, since it does not require culturing (Solera et al., 2001).

In order to determine specific methanogenic activity, different techniques have been developed by a number of researchers (Ince et al., 1995). The available techniques to monitor any changes in the numbers or activities of the methanogenic microorganisms in the digester include microscopic count, most probable number (MPN), ATP, coenzyme F_{420}, dehydrogenic activity, and specific methanogenic activity (SMA) under uncontrolled conditions.

Methanogenic activities are normally calculated by comparing the rate at which the substrate is consumed, or the amount of methane that is generated by the process, with the total biomass contained in the system. However, the results obtained with the parameter most commonly used in the determination of biomass, volatile solids (VS), are not always representative of the minority groups involved in the anaerobic treatment process (Solera et al., 2001). Such is the case with the methanogenic population. This implies a limitation in the usefulness of the activity measurements recorded by these means.

Applications of molecular based methods to studies of microbial community structure have eliminated some of these problems because these techniques have allowed the direct identification and enumeration of microbial populations in complex environments (Macario and Conway de Macario, 1988; Ward et al., 1992; Ahring, 1995; Amann et al., 1995; Raskin et al., 1996; Head et al., 1998; Davenport et al., 2000; Hugenholtz et al., 2001; Davenport and Curtis, 2004; Zheng et al., 2006). Whole cell fluorescence *in situ* hybridization (FISH) is a technique that uses fluorescently labelled phylogenetic oligonucleotide probes to detect specific whole cells/organisms in biological samples. DeLong et al. (1989) first demonstrated its use with bacteria. Raskin et al. (1994a,b) have used FISH to identify and quantify species and genus of methanogens present in anaerobic reactors. It can be a valuable tool for the study of microbial dynamics in natural environments (Hugenholtz et al., 2001; Davenport and Curtis, 2004). However, it must be kept in mind that physiological state is operationally defined and based on the general properties of a particular stain, and dormant or extremely slow-growing cells cannot be detected. The main advantages and disadvantages of FISH have been summarized by Sanz and Köchling (2007).

In the case of activity analyses, the tests used have permitted the evaluation of the maximum activity attainable by these microorganisms under standard test conditions, and these do not necessarily coincide with those in the treatment unit itself (Lazarova and Manem, 1995). The main advantage of *in situ* activity measurements in the reactor is that these were not carried out under standard conditions, obtaining specific methanogenic activity in the selected operational conditions as for example in each of the loading organic rates and/or retention times.

In this study, the methanogenic activity in thermophilic-dry anaerobic reactors has been determined by comparing the amount of methane generated in the system with the size of the methanogenic population, as determined by fluorescent *in situ* hybridization, from the start-up to the stabilization periods. These activity measurements have then been compared with those obtained by more classic means (relative to volatile solids).

2. Materials and methods

2.1. Experimental equipment

A laboratory-scale continuously stirred tank reactor (CSTR) without recycling of biomass and a working volume of 4.5 L was used for the anaerobic digestion of the organic fraction of municipal solid waste (OFMSW). The reactor temperature was maintained at 55 °C and the biogas generated was collected in a Tedlar bag (40 L). A shaking stick was used to mix and homogenize the liquid in the system (13 rpm). The pH was controlled by an on/off controller. The pH control was achieved using 5N NaOH and 1N H_3PO_4 solutions, which were added at a rate of 0.2 L/h by a peristaltic pump, when necessary. The initial pH of OFMSW-Inoculum was 7.2. The range of operation of the pH controller was between 6.5 and 8.5. These values are appropriate for methanogenic populations (Chynoweth and Isaacson, 1987; Hobson and Wheatley, 1993; Vavilin et al., 1998).

The reactor was operated with retention times (RT) in the range 40–25 days. The organic loading rate (OLR) added to the system was modified, but a constant OLR was maintained for each RT value. Four different organic loading rates were used ranging from 4.42 to 7.50 kg VS m^{-3} day^{-1}.

2.2. Inoculum and Feed solution

The reactor was loaded with 1.5 kg of milled dry synthetic OFMSW (90% TS). The moisture was adjusted using an inoculum from SEBAC (Sequencial Batch Anaerobic Composting) that consisted of a 1:1 v/v mixture of thermophilic sludge and leachate (Wang et al., 1999; Lin and Lee, 2002; Kim et al., 2003). A synthetic feed based on the nutritional requirements of the main populations of microorganisms involved was prepared (Martin et al., 1999). This type of feed avoids the problem of large variations in the composition of the selected OFMSW source.

The feed was added in a semi-continuous mode at one dose per day. Control of the total solids concentration of the feed is necessary to obtain a suitable level of performance for the dry anaerobic digestion. Therefore, pre-treatment of the OFMSW samples was necessary to adjust them to the required optimum values. The samples were dried at 55 °C for 48 h and then at ambient temperature for 72 h, until final moisture content of 10% was achieved. The dried OFMSW was milled until a particle size of approximately 1 cm was obtained and, finally, the moisture was adjusted to 70–75% (25–30% TS, which is characteristic of dry anaerobic digestion) with water, leachate from garbage, sludge, or combinations of these.

2.3. Reactor performance analysis

Control of the reactor was assessed by determining the following parameters: the volume and composition of the biogas (H_2, O_2, N_2, CH_4, and CO_2), total solids (TS), total volatile solids (TVS), pH, and total organic carbon (TOC). The analytical techniques were performed in accordance with the procedures described by Standard Methods (APHA, 1990) and Alvarez-Gallego (2005). The volume of biogas accumulated in the Tedlar bag was measured by a gas flow meter and gas composition was determined by gas chromatography (SHIMADZU GC-14 B) using a stainless steel column packed with Carbosieve SII (0.32 cm diameter and 2 m length), and a thermal conductivity detector. The injected sample volume was 1 mL and operational conditions were as follows: 7 min at

55 °C, ramped up at 27 °C/min to 150 °C; detector temperature of 255 °C; and injector temperature of 100 °C. The carrier gas was helium and the flow rate used was 30 mL/min. A standard gas (from Carburos Metálicos, S.A.: 4.65% H_2; 5.33% N_2; 69.92% CH_4; and 20.10% CO_2) was used for calibration of the system.

2.4. Quantification of methanogens by fluorescence in situ hybridization

The main steps of fluorescence *in situ* hybridization (FISH) of whole cells using 16S rRNA-targeted oligonucleotide probes are cell fixation, consequent permeabilization, and hybridization with the desired probe(s).

Biomass was sampled periodically from the thermophilic-dry anaerobic reactor into sterile universal bottles and immediately stored at −20 °C until they were fixed, as described in the following section (generally within 2 weeks). In order to collect a representative sample of the entire culture contents, the slurry was homogenized with a magnetic stirrer while samples were withdrawn with a 10-mL gas-tight syringe. Absolute ethanol was added to the bottles in a volume ratio of 1 sample to 1 ethanol.

The technique used for fixation and permeabilization of cells was based on the one used by Amann et al. (1990a). Previously, the samples were pre-treated. The most appropriate pre-treatment used for microbiological count of high solids content samples was the addition of Tween 80 and 120 sec of shaking (Yoon and Rosson, 1990; Heldal et al., 1996; Montero, 2006). A pre-treated OFMSW sample (1 mL) was transferred to a 1.5-mL eppendorf tube and centrifuged at 13,000*g* for 3 min. The supernatant was removed and the sample was washed using 1 mL of phosphate buffer saline (PBS). The sample was vortexed and centrifuged at 13,000*g* for 3 min. The supernatant was removed and the pellet was resuspended in 0.25 mL of PBS and 0.75 mL of 4% paraformaldehyde fixative solution. The sample was then incubated at 4 °C overnight. Before hydridization, the fixed samples were diluted with PBS (pH 7.2). Cell samples for *in situ* hybridization with a family *Methanobacteriaceae* specific probe (MB1174) were treated by a freeze thawing method to improve probe permeability into the cells (Sekiguchi et al., 1999).

Fixed cells (10 μL of sample/well) were spotted onto gelatin coated slides, air-dried at 37 °C for 20 min, and dehydrated by serial immersion of the slides in 50%, 80% and 96% (v/v) aqueous ethanol solutions for 3 min each (Amann et al., 1992). For each hybridization, 8 μL hybridization solution (4.5 M NaCl, 200 mM Tris–HCl [pH 7.2], 10% SDS, and formamide [for theoretical concentrations for each probe, see Table 1]), and 2 μL of probe solution (50 ng/μL) were applied to each well of the slides. The microorganisms in anaerobic reactors belong to three domains: *Bacteria*, *Archaea*, and *Eucarya*. *Bacteria* are the majority of the microorganisms in the reactors, *Archaea* are present in smaller amounts, and *Eucarya* are present at very low levels (below 1% in most cases), which indicates that anaerobic protozoa likely are not abundant in anaerobic digesters (Griffin et al., 1998). The following 16S rRNA-targeted oligonucleotide probes were used in this study: *Bacteria*-universal probe EUB338 (Amann et al., 1990a,b), *Archaea*-universal probe ARC915 (Stahl and Amann, 1991); H_2-utilizing methanogens probe MB1174 (specifically *Methanobacteriaceae*) (Raskin et al., 1994a,b; Sekiguchi et al., 1999), which are the main hydrogen scavengers during start-up of thermophilic-dry anaerobic reactors (Griffin et al., 1998); and acetate-utilizing methanogens probe MX825 (specifically *Methanosaeta*) (Raskin et al., 1994a,b). The genus *Methanosaeta* use only an acetate-like characteristic substrate, while *Methanosarcina* use acetate and other substrates (H_2–CO_2; methanol and methylamines) (Raskin et al., 1994a,b; Griffin et al., 1998). Thus, it had been shown that digesters that started up successfully contained high levels of *Methanosaeta* (McHugh et al., 2003; McMahon et al., 2004; Pender et al., 2004). All probes were labelled with 6-FAM at the 5′ terminal, except S-D-bact-0338-a-S-18 labelled with Cy3. The slides were incubated at the appropriate temperature for the probe (usually 46 °C) for 1.5 h in an isotonic moisture chamber.

After hybridization, the slides were flushed with 2 mL of wash solution (4.5 M NaCl, 200 mM Tris–HCl (pH 7.2), 10% SDS, and 0.5 M EDTA (pH 8.0)) and were immediately immersed in enough volume of the same wash solution so as to cover the wells of interest (e.g., approximately 50 mL in an universal tube) at 48 °C for 15 min. This step was repeated. Finally, the slides were rinsed thoroughly with milliQ water and were air-dried for 5 min. These slides may be stored in the dark at −20 °C, or viewed immediately.

For viewing, the slides were mounted in a small drop of the antifadant Citifluor (AF1, Canterbury, UK) prior to the addition of a cover glass. When an oil-immersion lens is to be used during microscopy, the edges of the cover glass should be coated in colourless nail varnish to prevent movement of the slide and evaporation of the Citifluor. The slides prepared in this manner can be stored in the dark at 4 °C. If the slide is not viewed using an oil-immersion lens, the cover glass may be removed after viewing, the Citifluor can be rinsed away with milliQ water, and the slide air-dried prior to storage in the dark at −20 °C.

The samples were examined visually and cells counted using a Nikon Labophot-2A/2 epifluorescence microscope fitted with a 100 W mercury lamp and an ×100 oil objective. The filters used were B-2A (DM 510, excitation 450–490 and Barrer 520) for 6-FAM labelled probes and G-2A (DM580, excitation 510–560 and Barrer 590) for the Cy3 labelled probe. Digital images of the slides were taken with a digital camera. For each sample, 20 randomly selected fields of view were counted (Kepner and Pratt, 1994). The number of cells per millilitre was then determined from values for: the area of the sample spot; the area of field of view (FOV); the volume and the dilution of sample applied; and the original volume of the sample used for hybridization, as follows: total number of cells milliliter^{-1} = (number of cells per FOV × area of sample spot)/(area of FOV × volume applied × dilution × original sample volume) (Davenport and Curtis, 2004). The sum of *Eubacteria* and *Archaea* is 100%. Thus, from the calculated percentage of *Archaea*,

Table 1
Oligonucleotides probes used in this study, classified according to the oligonucleotide probe database (Alm et al., 1996)

Probes	Position	Probe sequences (from 5′ to 3′)	Target	Form amide (%)	Temperature (°C)	References
S-D-Bact-0338-a-A-18	338–355	GCTGCCTCCCGTAGGAGT	*Eubacteria*	20	46	Amann et al. (1990b)
S-D-bact-0338-a-S-18	338–355	ACTCCTACGGGAGGCAGC	None (negative control)	20	46	Amann et al. (1990a)
S-D-Arch-0915-a-A-20	915–934	GTGCTCCCCCGCCAATTCCT	*Archaebacteria*	35	46	Stahl and Amann (1991)
S-F-Mbac-1174-a-A-22	1174–1195	TACCGTCGTCCACTCCTTCCTC	*Methanobacteriaceae* (H_2-utilizing methanogens)	35	46	Raskin et al. (1994a,b), Sekiguchi et al. (1999)
S-F-Msae-0825-a-A-23	825–847	TCGCACCGTGGCCGACACCTAGC	*Methanosaeta* (Acetate-utilizing methanogens)	20	46	Raskin et al. (1994a,b)

the percentages of H_2-utilizing methanogens and acetate-utilizing methanogens could be determined.

2.5. Determination of biomass

Biomass was determined by measuring the total volatile solids contained in the digester medium, according to 'standard methods' (APHA, 1990).

3. Results and discussion

3.1. Reactor performance and methanogenic population evolution

Performance and operating parameters for the control of the anaerobic process are shown in Table 2 (Fernández-Güelfo et al., 2005). The cellular concentration and percentages, respectively, of *Eubacteria*, *Archaea*, H_2-utilizing methanogens, and acetate-utilizing methanogens were obtained by fluorescent *in situ* hybridization are shown in Tables 3 and 4.

Figs. 1 and 2 represent the evolution of the composition of biogas and the dynamics of methanogenic population, respectively. Even though significant levels of methanogens were present in the thermophilic digester, they were apparently not able to adjust within one day to operating conditions, as demonstrated by the low methane levels in the biogas in the first stage (OLR_0 = 4.42 kg VS $m^{-3}day^{-1}$; see Table 2 and Fig. 2). The level of acetate-utilizing methanogens decreased during the first few days, indicating that the removal rate through wash-out was greater than their growth rates. The total methanogen concentrations remained relatively constant during the first few days of operation, because the loss in acetate-utilizing methanogens was compensated by an increase in the H_2-utilizing methanogens level (see Fig. 2). The latter apparently served as the main hydrogen scavengers during this period of rapidly increasing activity, reflected by rapid increases in the gas production rate and the level of methane in the biogas during the next stage (OLR_0 = 5.07 kg VS m^{-3} day^{-1}; see Table 2).

H_2-utilizing methanogens are the most abundant *Archaea* during the start-up stage because of the greater amount of hydrogen generated in this period (OLR_0 = 4.42 kg VS m^{-3} day^{-1}, see Tables 2 and 4, Figs. 1 and 2). Thus, in this stage, the hydrolysis and acidogenesis of waste were carried out, producing large amounts of hydrogen and increasing its partial pressure. This indicates that acidogenic bacteria produce volatile fatty acids while the partial

Table 4
Percentages of *Eubacteria*, *Archaea*, H_2-utilizing methanogens and acetate-utilizing methanogens in the OFMSW reactor.

OLR_0 (kg VS $m^{-3}day^{-1}$)	*Eubacteria* percentage (%)	*Archaea* percentage (%)	H_2-utilizing methanogens percentage (%)	Acetate-utilizing methanogens percentage (%)
4.42	87.58 ± 32.01	12.42 ± 7.10	11.11 ± 5.56	1.32 ± 5.28
5.07	67.43 ± 16.74	32.57 ± 17.31	8.58 ± 3.34	23.99 ± 16.91
5.92	60.66 ± 7.89	39.34 ± 8.61	6.78 ± 2.12	32.56 ± 7.01
7.50	60.41 ± 15.10	39.59 ± 8.57	7.19 ± 1.39	32.40 ± 7.81

Table 2
Performance and operating parameters for the control of the anaerobic process during start-up and stabilization stages (Fdez.-Güelfo, 2005)

OLR_0 (kg VS m^{-3} day^{-1})	Elim.VS (%)	Biogas (m^3 m^{-3} day^{-1})	Methane (m m^{-3} day^{-1})	Methane yield coefficient (m^3CH_4 kg COD^{-1} d^{-1})	Composition of biogas (%)		
					H_2	CH_4	CO_2
4.42	67.14 ± 1.72	0.47 ± 0.70	0.01 ± 0.01	0.02 ± 0.01	28.86 ± 20.72	10.62 ± 18.67	60.52 ± 28.45
5.07	75.43 ± 2.74	1.93 ± 1.27	0.47 ± 0.31	0.43 ± 0.29	6.59 ± 10.01	25.34 ± 12.97	68.07 ± 7.72
5.92	81.51 ± 2.29	1.15 ± 0.40	0.51 ± 0.21	0.34 ± 0.14	n.d.	49.96 ± 8.05	50.04 ± 8.05
7.50	86.87 ± 2.87	1.53 ± 0.27	0.57 ± 0.08	0.30 ± 0.04	n.d.	40.86 ± 4.53	59.14 ± 4.53

OLR_0 = organic load rate; the food of the reactor, expressed in kg VS per m^3 of reactor per day. Elim. VS = organic removal efficiency as a percentage of VS elimination. Biogas: volume of gas generated as m^3 of biogas per m^3 of reactor per day. Methane: volume of methane generated as m^3 of methane per m^3 of reactor per day. n.d.: not detected.

Table 3
Cellular concentration of *Eubacteria*, *Archaea*, H_2-utilizing methanogens and acetate-utilizing methanogens in the OFMSW reactor

OLR_0 (kg VS m^{-3} day^{-1})	*Eubacteria* (Cell g TVS^{-1}) ($\times 10^{-9}$)	*Archaea* (Cell g TVS^{-1}) ($\times 10^{-9}$)	H_2-utilizing methanogens (Cell g TVS^{-1}) ($\times 10^{-9}$)	Acetate-utilizing methanogens (Cell g TVS^{-1}) ($\times 10^{-9}$)
4.42	0.93 ± 0.34	0.14 ± 0.08	0.12 ± 0.06	0.01 ± 0.04
5.07	1.45 ± 0.36	0.79 ± 0.42	0.18 ± 0.07	0.61 ± 0.43
5.92	1.46 ± 0.19	0.96 ± 0.21	0.16 ± 0.05	0.79 ± 0.17
7.50	2.64 ± 0.66	1.71 ± 0.37	0.31 ± 0.06	1.41 ± 0.34

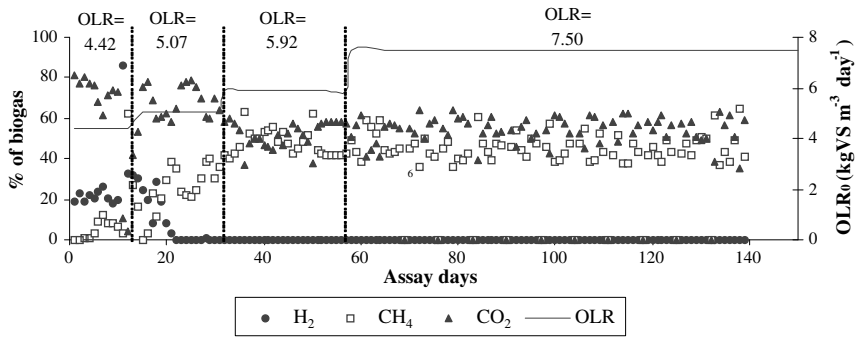

Fig. 1. Evolution of composition of biogas in the OFMSW reactor.

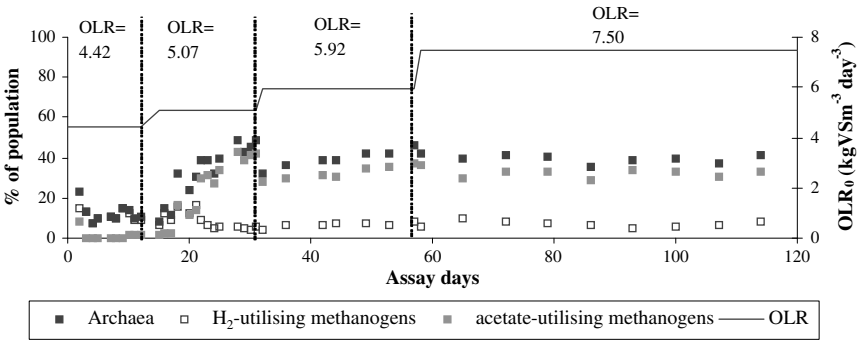

Fig. 2. Evolution of *Archaea*, H_2-utilisng methanogens and acetate-utilizing methanogens in the OFMSW reactor.

pressure of hydrogen is high and CO_2/H_2 is low, whereas homoacetogenic bacteria synthesize acetate from H_2 and CO_2, operating in a manner complementary to H_2-utilizing methanogens. The acetogenic stage begins when the partial pressure of hydrogen decreases (Boone, 1987). In this stage, the acetogenic bacteria synthesize acetate. Of the total methane synthesized in the anaerobic reactors, 54% comes from acetate and hydrogen (Stronach et al., 1986). In a similar fashion, H_2-utilizing methanogens consume hydrogen, decreasing its partial pressure. The major portion of methane generated during the start-up stage was produced by H_2-utilizing methanogens, since acetate-utilizing methanogens do not grow very well in presence of hydrogen in the system. For this reason, acetate-utilizing methanogens are practically nonexistent during this period. Thus, the relation between H_2-utilizing methanogens and acetate-utilizing methanogens is 11:1 during the start-up stage. An increase of the organic loading rate (OLR_0 = 5.07 kg VS m^{-3} day^{-1}) and a change of the hydrogen level is accompanied by a decrease of H_2-utilizing methanogens and an increase of acetate-utilizing methanogens (see Tables 2 and 4, Figs. 1 and 2). Thus, the ratio of H_2-utilizing methanogens to acetate-utilizing methanogens at an OLR_0 of 5.07, 5.92, and 7.50 kg VS m^{-3} day^{-1} is 9:24, 7:33, and 7:32, respectively. Nevertheless, H_2-utilizing methanogens do not disappear completely in stable stages of anaerobic digestion, but are present in low abundance during steady-state (see Fig. 2). These results are in agreement with those obtained by McMahon et al. (2001, 2004) in stable anaerobic digesters.

3.2. Methanogenic activity analysis

The average results of *Archaea* concentration (cell mL^{-1}), biomass (TVS), and activity in each of the organic loading rates studied are shown in Table 5.

In the start-up stage, an increase in the OLR (from 4.42 to 5.07 kg VS m^{-3} day^{-1}) is accompanied by an increase in the *Archaea* concentration and a decrease in total volatile solids content, as well as an increase in methanogenic activity with respect to both biomass and the concentration of methanogens. In this period the methanogenesis of accumulated substrate during the incubation period (OLR_0 = 4.42 kg VS m^{-3} day^{-1}) begins. Subsequently, a slight decrease in the *Archaea* concentration is observed since in systems with no biomass retention, increased OLR is reflected by faster rates of dilution and, as a result, in a greater number of microorganisms leaving the system daily in the effluent. Consequently, a larger amount of substrate is consumed in the anabolic route, in which no methane is generated, in order to keep the size of the population at a steady-state. The total methanogenic activity

Table 5
Archaea concentration, biomass and activity of methanogens in the OFMSW reactor

RT (days)	OLR_0 (kg VS m^3 day^{-1})	*Archaea* (Cell mL^{-1}) ($\times 10^{-8}$)	TVS (g mL^{-1})	Activity[a] ($mL_{methane}$ $cell^{-1}day^{-1}$) ($\times 10^{-8}$)	Activity[b] ($mL_{methane}$ g $TVS^{-1}day^{-1}$)
40	4.42	0.32 ± 0.22	0.23 ± 0.01	0.04 ± 0.03	0.04 ± 0.04
35	5.07	1.31 ± 0.65	0.17 ± 0.02	0.23 ± 0.14	2.76 ± 1.76
30	5.92	1.24 ± 0.25	0.13 ± 0.02	0.33 ± 0.07	3.87 ± 1.45
25	7.50	1.50 ± 0.16	0.09 ± 0.02	0.38 ± 0.06	6.46 ± 1.39

OLR_0 = organic load rate; the food of the reactor, expressed in kg VS per m^3 of reactor per day.
TVS = total volatile solid and RT = retention time.
[a] Methanogenic activity determined with reference to *Archaea* concentration and daily methane production.
[b] Methanogenic activity determined with reference to biomass (total volatile solids) and daily methane production.

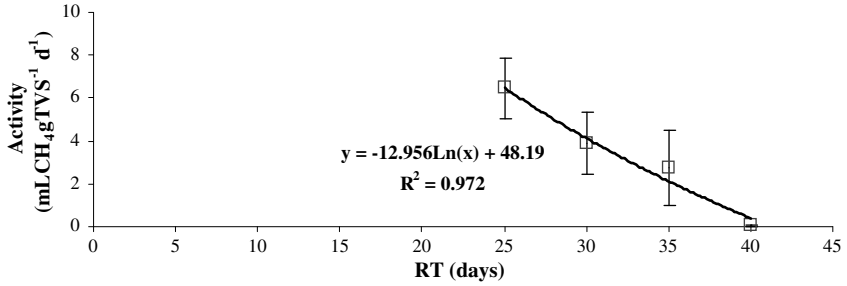

Fig. 3. Correlation between the retention time to the methanogenic activity with respect to biomass (total volatile solid) in the thermophilic-dry anaerobic reactor.

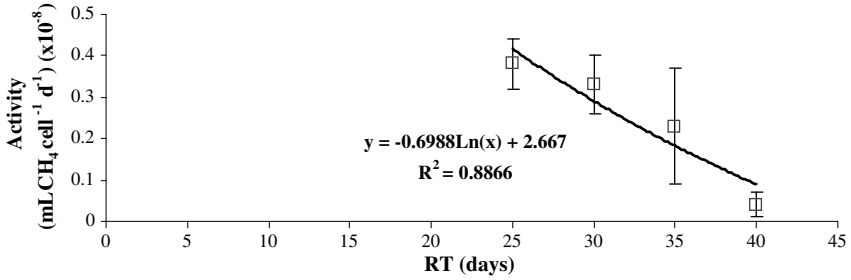

Fig. 4. Correlation between the retention times to the methanogenic activity with respect to total concentration of methanogens (*Archaea*) in the thermophilic-dry anaerobic reactor.

increases slightly to stabilize in the last period (OLR_0 = 7.50 kg VS m^{-3} day^{-1}).

Attempts were made to correlate the retention time with the activity of the biomass (total volatile solids) and to total methanogenic population (*Archaea*) present in the system studied. In both cases, a logarithmic relationship is obtained (see Figs. 3 and 4). A decrease in retention time is accompanied by an increase in methanogenic activity with respect to both biomass and the total concentration of methanogens. A greater number of microorganisms left the system daily because of the decreased retention time. The population inside the reactor was renovated, becoming younger and therefore more active.

Table 6 shows the average results in respect of H_2-utilizing and acetate-utilizing methanogens concentration (cell mL^{-1}) and specific methanogenic activity in each of the organic loading rates studied. Methane production in the first retention period (40 days) must be fundamentally due to H_2-utilizing methanogens population. During the rest of the stages, methane production is due to H_2-utilizing and acetate-utilizing methanogens. The methane production rates in these retention periods (35, 30, and 25 days) were corrected by a multiplying factor of 0.70 in the case of acetate-utilizing methanogens and 0.30 for H_2-utilizing methanogens since approximately 70% of the methane formed during the anaerobic digestion of complex substrate results from acetic acid and 30% from hydrogen and carbon dioxide (Zinder, 1993; Ince et al., 1995). Fig. 5 shows the evolution of specific methanogenic activity along the assay.

In the first stage (OLR_0 = 4.42 kg VS m^{-3} day^{-1}), at approximately day-7 the percentage of methane increased while the percentage of hydrogen and carbon dioxide decreased, indicating a slight increase in the activity of H_2-utilizing methanogens (see Figs. 1 and 5). In the second period (OLR_0 = 5.07 kg VS m^{-3} day^{-1}), the methanogenic activity of H_2-utilizing methanogens increased, consuming the hydrogen and decreasing its partial pressure. This initiated acetoclastic methanogenesis, as observed in the increase of methanogenic activity of acetate-utilizing methanogens (see Fig. 5). This augmented the methane progressively until it stabilized in the last two retention times (see Fig. 1). In the stabilization period (OLR_0 = 7.50 kg VS m^{-3} day^{-1}), methanogenic activity of both populations was stable. The methanogenic activity of H_2-utilizing methanogens was higher than acetate-utilizing methanogens at an OLR of 7.50 kg VS m^{-3} day^{-1}. This maintained a low partial pressure of hydrogen, which is necessary to prevent inhibition of acetate-utilizing methanogens. The growth rate of H_2-utiliz-

Table 6
H_2-utilizing and acetate-utilizing methanogens concentration and specific methanogenic activity in the OFMSW reactor

RT (days)	OLR_0 (kg VS m^3 day^-)	H_2-utilizing methanogens (Cell mL^{-1}) ($\times 10^8$)	Acetate-utilizing methanogens (Cell mL^{-1}) ($\times 10^8$)	Activity[a] ($mL_{methane}$ $cell^{-1}day^{-1}$)($\times 10^{-8}$)	Activity[b] ($mL_{methane}$ $cell^{-1}$ day^{-1}) ($\times 10^{-8}$)
40	4.42	0.28 ± 0.16	0.04 ± 0.09	0.04 ± 0.03	–
35	5.07	0.31 ± 0.14	0.99 ± 0.67	0.44 ± 0.27	0.21 ± 0.13
30	5.92	0.21 ± 0.06	1.03 ± 0.19	0.74 ± 0.28	0.35 ± 0.15
25	7.50	0.27 ± 0.04	1.23 ± 0.17	0.64 ± 0.09	0.33 ± 0.06

OLR_0 = organic load rate; the food of the reactor, expressed in kg VS per m^3 of reactor per day.
TVS = total volatile solid and RT = retention time.
[a] Methanogenic activity determined with reference to H_2-utilizing methanogens concentration and daily methane production.
[b] Methanogenic activity determined with reference to acetate-utilizing methanogens concentration and daily methane production.

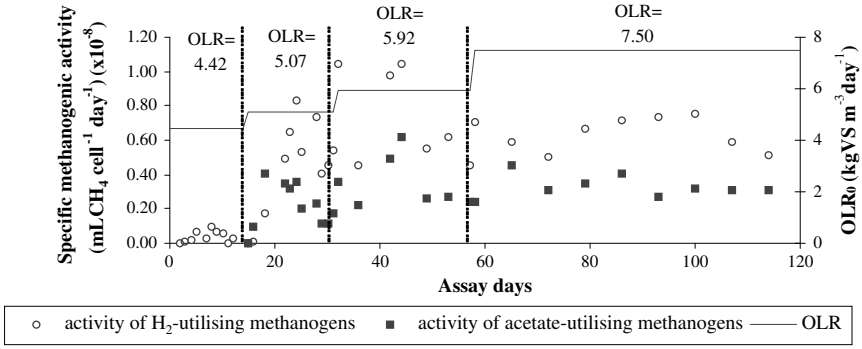

Fig. 5. Evolution of the specific methanogenic activity along the assay.

ing methanogens is faster than acetate-utilizing methanogens. Therefore, it is logical that the H_2-utilizing methanogens were more active.

The main advantage of this study is the method used to calculate methanogenic activity. This form has allowed obtaining a direct measurement of activity, compared to activity tests, which represented an indirect measurement of the maximum activity. The application of a molecular technique (FISH) has enabled the principal methanogenic populations to be distinguished, and to obtain a specific measurement of activity without the need to extrapolate the sample to activity tests.

4. Conclusion

Previous research has emphasized the importance of microbial community characterization for optimal digester performance, mainly the physiological state (activity) (Sorensen and Ahring, 1993; Ince et al., 1995; Solera et al., 2001). In this study, it is observed that the evolution of physical and chemical parameters is intensely related to the dynamic of microbial populations.

Total methanogenic activity may be determined with reference to either total methanogenic concentration or to TVS values. This was shown by means of the high correlation presented between the activity and the retention time (R^2 0.8866 and R^2 0.9720, respectively). Nevertheless, the methanogenic activity measurements determined with respect to methanogenic concentration provide more specific information regarding the physiological state of this population than regarding total biomass. The methanogenic concentration derived by FISH has represented a direct measurement of the methanogenic biomass, compared to VS, which provides a measurement of both non-methanogenic and methanogenic biomass.

The fluorescent *in situ* hybridization technique employed in this study has permitted the quantification of the specific methanogenic activity of the main methanogenic groups (H_2-utilizing and acetate-utilizing methanogens). The evolution of these is closely related to the evolution of biogas composition in the anaerobic reactor studied, distinguishing between the production of methane as a result of hydrogen consumed or as a result of acetate consumed.

This study has demonstrated that the elevated methanogenic activity of H_2-utilizing methanogens has allowed the reactor to maintain a low partial pressure of hydrogen. This condition has favoured the development of a thermophilic-dry anaerobic process.

Since the activity is calculated *in situ* without extrapolating the effluent of the reactor, it is a clear advantage over the activity test, since it permits direct measurement of the specific activity.

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