

INFLUENCE OF THE INOCULUM SOURCE AND CHARACTERISTICS ON THE METHANE YIELD COEFFICIENT, KINETICS AND MICROBIAL COMMUNITIES INVOLVED IN THE ANAEROBIC DIGESTION PROCESS OF SUNFLOWER OIL CAKE IN BATCH MODE

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INTRODUCTION

Sunflower oil cake (**SuOC**) is the solid waste generated in the sunflower oil extraction process. Great quantities (4-5 million tons) of this waste are produced every year in Spain.

The **anaerobic digestion** (AD) is a process performed by complex groups of microorganisms (**Bacteria and Archaea**) that allow to obtain from organic residual wastes an added value product as the **biogas** (mixture methane/carbon dioxide) with high energetic value (21.4 MJ per m³).



INTRODUCTION

In this study, the biochemical methane potential (**BMP**) or maximum quantity of methane that a substrate can produce was studied for SuOC under the action of three different inocula.

The initial **microbial communities** that are present in the anaerobic inocula can be different depending on the **source of the sludges**. The study of the community fingerprints allow to find significant differences between the inoculum communities and their development after the AD process of an initial waste.



Sunflower oil cake (SuOC)

OBJECTIVE

There are only a few studies that have had into account the influence of the inoculum source in the AD systems. Most of the times the AD processes are carried out taking an inoculum sludge with unknown microbial communities from: landfill leachate, anaerobic digesters already working, rumen, sewage, etc.

The **objective of this work** was to study the influence of the inoculum source and its microbial composition on the SuOC anaerobic digestion. With this purpose a **BMP test** of this substrate was evaluated for **three different inocula** in the same conditions. The influence of the inocula on the methane yield coefficient and the kinetic constants of the process was also evaluated. Finally, a study of the **community fingerprints** for finding significant differences between the inoculum and the developed communities after the AD process was also carried out.



MATERIAL AND METHODS

The solid substrate used for the experiments was the waste resulting from the sunflower oil extraction process (**SuOC**). The study was carried out with the most abundant size range of particles found in the sunflower oil cake waste (0.7-1.0 mm). **Table 1** shows the full composition and main features of the SuOC.

| Parameter | Value |
|--|-------|
| Moisture (%) | 7.0 |
| Total protein (%) | 23.4 |
| Fats (%) | 1.4 |
| Carbohydrates (%) | 58.7 |
| Hemicellulose (%) | 9.2 |
| Lignin (%) | 9.5 |
| Cellulose (%) | 21.7 |
| TS (%) | 93 |
| MS (%) | 6.5 |
| VS (%) | 86.5 |
| TCOD (g O ₂ g ⁻¹ TS dry basis) | 1.15 |

^a Mean values are averages of four determinations (The standard deviations were ≤ 0.05).
^b TS: total solids, MS: mineral solids, VS: volatile solids, TCOD: total chemical oxygen demand.

MATERIAL AND METHODS

Three different inocula were used for the experiments:

- **Sludge I:** an anaerobic granular sludge derived from a 450 m³ full-scale upflow anaerobic sludge blanket (UASB) reactor treating wastewaters coming from a soft drink elaboration industry.
- **Sludge II:** a flocculent anaerobic sludge coming from a 2000 m³ completely stirred tank reactor (CSTR) treating sewage sludge from a conventional urban wastewater treatment plant.
- **Sludge III:** an anaerobic granular sludge from a 550 m³ UASB reactor treating brewery wastewaters.

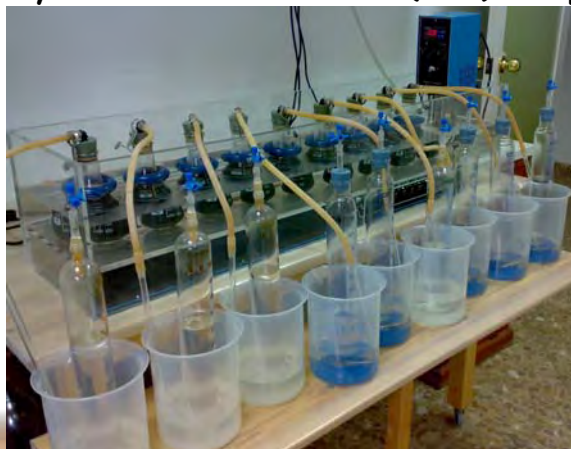


MATERIAL AND METHODS

Table 2. Characteristics of the three sludges used for the experiments.

| Sludge | pH | TS (g/L) | VS (g/L) |
|--------|-----|----------|----------|
| I | 7.4 | 30 | 25 |
| II | 7.6 | 43 | 20 |
| III | 7.5 | 83 | 47 |

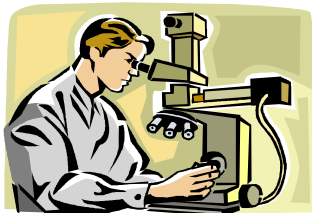
The experiments were carried out in a **thermostated water bath multireactor system (at 35 °C)** and in **batch mode**. The reactors were magnetically stirred at 250 rpm. All the BMP assays were carried out in triplicate. In every run two controls without substrate addition were used. The reactors were filled with: 15 g VS L⁻¹ of sludge, the corresponding quantity of SuOC until an **inoculum-substrate ratio of 2**, 25 mL of a 50 g NaHCO₃ L⁻¹ solution to keep the alkalinity in a correct value, 50 mL of a nutrient solution and distilled water until a total volume of 250 mL. Methane generation was measured by a NaOH solution (2N) displacement during 12 days.



MATERIAL AND METHODS

Solids, total chemical oxygen demand and total protein were determined according to standard methods. **Fat content** was extracted by a soxhlet system using hexane. **Cellulose, hemicellulose and lignin** were determined by Goering and Van Soest method.

Microbial communities were studied by molecular fingerprinting methods complemented with cloning and sequencing for the identification of the major components of the bacterial and archaeal communities. DNA was extracted using the Nucleospin Food DNA extraction kit according to the manufacturer's recommendations. Fragments of the 16S ribosomal RNA (16S rRNA) genes were amplified by PCR with different primer pairs depending on the use of the amplification products. Fingerprints of the bacterial and archaeal communities were obtained by **Denaturing Gradient Gel Electrophoresis (DGGE)**.



RESULTS AND DISCUSSION

Methane yields and specific rate constants

The best methane yield coefficients after 12 days were obtained for **sludge II and III (193 and 205 mL CH₄ accumulated/g VS added**, respectively), these yields were higher than that obtained for sludge I (156 mL CH₄ accumulated/g VS added). The value of this yield for the sludge III was 6.2 % higher than for sludge II and 23.7 % higher than for the sludge I. So, **sludge II and III had a similar methane yield and higher than that obtained for sludge I**. In addition, the percentage of volatile solids removed was 42 % for sludges II and III and only 33 % for sludge I.

RESULTS AND DISCUSSION

A first-order kinetic model (Roediger equation) was used to calculate the specific rate constant of the process:

$$G = G_m [1 - \exp(-k_o t)] \quad (1)$$

where:

- G is the volume of methane gas accumulated at a given time (mL).
- G_m is the volume accumulated at an infinite digestion time (mL).
- k_o (day^{-1}) is the observed specific rate constant and
- t is the digestion time (days).

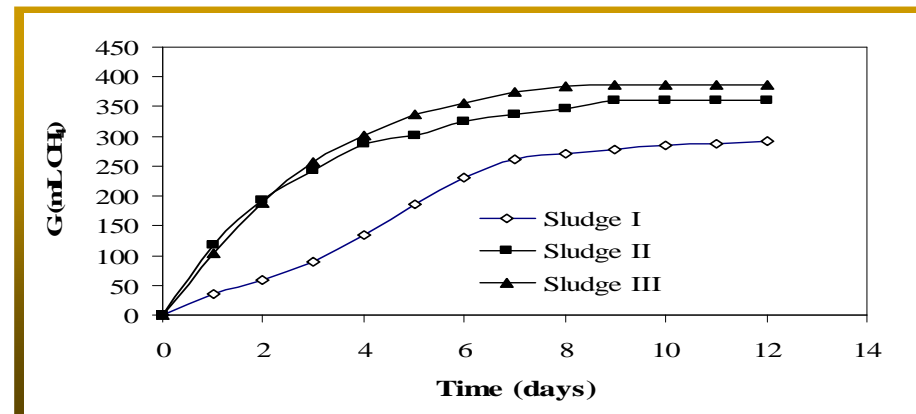


Fig. 1. Volume of methane accumulated for the sludge I, II and III.

RESULTS AND DISCUSSION

Table 3 shows the values obtained for G_m and k_o (\pm SD: standard deviation, VC: variation coefficient and R^2 : coefficient of determination).

| Table 3 | | | | | |
|---------|--------|---------------------------------------|---------------------------------------|-------------------|-------------------|
| Sludge | R^2 | $G_m \pm SD$ (mL CH ₄) | $k_o \pm SD$ (days ⁻¹) | VC_{G_m} (%) | VC_{k_o} (%) |
| I | 0.9675 | 425 \pm 65 | 0.11 \pm 0.02 | 15.2 | 25.1 |
| II | 0.9989 | 366 \pm 2 | 0.37 \pm 0.01 | 0.6 | 2.0 |
| III | 0.9968 | 403 \pm 4 | 0.33 \pm 0.01 | 1.1 | 3.7 |

G_m and k_o were obtained by **non-linear regression** using the software Sigmaplot 9.0. The best adjust of the experimental data to this first order model was obtained for sludges II and III.

Therefore, the specific rate constant of the **inoculum II was 12 % higher** than that achieved with the inoculum III and, **236 % higher** than that obtained with the inoculum I.

RESULTS AND DISCUSSION

Microbial communities study

Comparisons of fingerprints from bacterial and archaeal communities in the three inocula used in this study showed **significant differences among the communities in these three inocula**. Well developed communities (after AD process) showed no significant differences for bacterial communities. However, comparison of **archaeal community fingerprints showed significant differences between the inoculum and the developed communities after the AD of SuOC**.

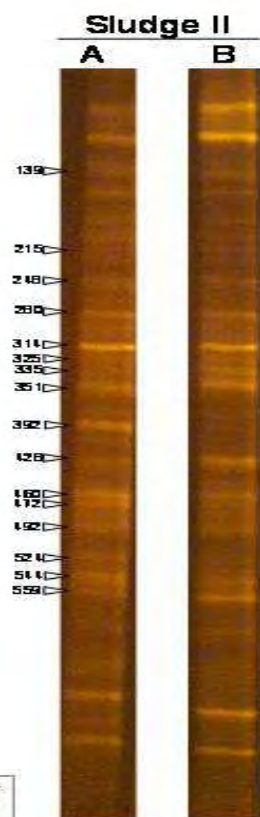
Despite of this change in the structure of the archaeal communities, **the major microbial components in the inocula remained as final communities** after the AD process. As well, the archaeal community fingerprints from the different inocula were significantly different ($P < 0.001$) and the final archaeal communities developed during the AD process conserved also significant differences ($P < 0.001$) as a result of the distinct used microbial inocula.



RESULTS AND DISCUSSION

The bacterial and archaeal communities from **sludge II**, the inoculum showing optimum methane kinetic parameters, were studied in further detail to identify the major components. Table 4 shows the proportion of the major bacterial constituents of the community in sludge II as determined through community fingerprinting analysis using PCR-DGGE from sludge II.

Table 4



| Migration | Taxonomic affiliation (Accession No. of closest homologue) | Fraction inoculum ¹ | Fraction BMP ¹ |
|-------------------------|---|-----------------------------------|------------------------------|
| 139 | Chloroflexi (CU926181) | 3.4 | 3.8 |
| 215 | Betaproteobacteria (GU454925) | 1.9 | 0.8 |
| 248 | Candidate Division WS6 (AF423183) | 3.4 | 1.6 |
| 280 | Chloroflexi (EF174275) | 3.0 | 2.7 |
| 314 | Chloroflexi (CU924314) | 6.6 | 5.9 |
| 325 | Actinobacteria (AY426438) | 2.0 | 1.3 |
| 335 | Alphaproteobacteria (AJ440751) | 1.2 | 3.8 |
| 351 | Alphaproteobacteria (GQ500763) | 5.3 | 6.7 |
| 392 | <i>Thauera</i> , Betaproteobacteria (DQ098974) | 5.6 | 1.0 |
| 428 | Bacteroidetes (CU922674) | 2.7 | 6.1 |
| 460 | <i>Paracoccus</i> , Alphaproteobacteria (FJ386516) | 5.7 | 4.8 |
| 472 | Chromatiales, Gammaproteobacteria (AM176837) | 4.4 | 1.5 |
| 492 | Thermoanaerobacteriales, Firmicutes (EU878332) | 2.1 | 2.5 |
| 524 | <i>Synergistes</i> , Synergistetes (FN436049) | 2.4 | 1.4 |
| 544 | Firmicutes (CU919983) | 6.9 | 3.8 |
| 559 | Bacteroidetes (AB330856) | 2.6 | 5.4 |
| Total identified | | 59.2 | 53.1 |

RESULTS AND DISCUSSION

The **Alphaproteobacteria** (20.6% and 28.8% of total identified DNA in the inoculum and after the AD, respectively), within the Family **Rhodobacteraceae** (e.g., *Paracoccus*), and the **Chloroflexi** (22.6% and 23.4% of total in the inoculum and in the community developed after the AD, respectively) were the dominant bacterial groups. The **Proteobacteria**, identified through members of the **Alphaproteobacteria**, **Betaproteobacteria** and **Gammaproteobacteria**, represented up to 40.7% and 35% of the identified bacteria in the inoculum and in the anaerobic digester, respectively. Other major bacterial groups identified in the community were **Bacteroidetes** (between 9.0% and 21.7% of identified bacterial phylotypes), **Firmicutes** (over 11%; e.g., *Thermoanaerobacterium*), **Actionobacteria** (3.4% to 2.5%), **Synergistetes** (e.g., *Synergistes*)(above 2%), and **Candidate Division WS6** (between 3.0% and 5.7% of the identified phylotypes).



RESULTS AND DISCUSSION

Table 5 shows the proportion of the major **archaeal phylotypes** in sludge II determined through community fingerprinting analysis using PCR-DGGE from sludge II. The detected sequences from the archaeal community were all corresponding to **methane-producing Archaea** and they belonged to the orders **Methanosarcinales** and **Methanomicrobiales**. The **Methanosarcinales**, mainly represented by the genus **Methanosaeta**, were the dominant methanogens (above 67% of the archaeal community).

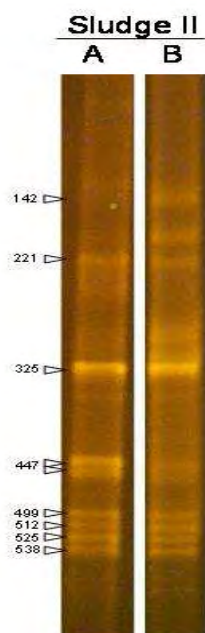


Table 5

| Migration | Taxonomic affiliation (Accession No. of closest homologue) | Fraction inoculum ¹ | Fraction BMP ¹ |
|-------------------------|---|-----------------------------------|------------------------------|
| 142 | Methanosarcinales (FJ705109) | 6.0 | 7.7 |
| 221 | <i>Methanosaeta</i> , Methanosarcinales (AB494241) | 12.1 | 7.0 |
| 325 | <i>Methanosaeta</i> , Methanosarcinales (FM162203) | 20.5 | 28.8 |
| 447 | Methanosarcinales (GU196156) | 16.9 | 11.4 |
| 499 | <i>Methanosaeta</i> , Methanosarcinales (EU591661)) | 6.4 | 6.3 |
| 512 | Methanosarcinales (CU916012) | 5.8 | 8.2 |
| 525 | Methanomicrobiales (EU591675) | 8.4 | 5.7 |
| 538 | Methanomicrobiales (EU591675) | 6.9 | 7.1 |
| Total identified | | 83.0 | 82.2 |

¹ Percentage of total fluorescence intensity quantified from the banding pattern of PCR-DGGE analysis

RESULTS AND DISCUSSION

The conservation of major components of the bacterial communities used to inoculate the bioreactors suggested the **importance of using a productive inoculum** to initiate AD processes and to reach optimal methane productions and maximum degradation of the substrate. **Only the archaeal community responsible for generating methane as final product of the AD showed a moderate level of selection** during the process although our results confirm that the major components of the archaeal communities present in the inoculum used to initiate the digestion process remain as major constituents of the archaea.

The major **bacterial components** found for the AD of this substrate (**Proteobacteria, Chloroflexi and Firmicutes**) were in agreement with the bacterial groups in communities reported during the digestion process of organic wastes.



RESULTS AND DISCUSSION

Among the **Archaea**, the microorganisms responsible for the production of methane that were detected during this study belonged to the orders **Methanosarcinales** (i.e., *Methanosaeta*) and **Methanomicrobiales** in agreement to previous reports on the AD of other substrates. **The principal groups of methanogens found were acetoclastic**. As a consequence, the major role of the bacterial community during this anaerobic process appeared to be the production of acetate which will lead the production of methane by Archaea. Bacterial communities are preserved through the AD of sunflower oil cake. This observation strongly suggests that the selection of an adequate inoculum microbial assemblage is required to reach an optimum transformation of the substrate and maximum production of methane in the shortest time possible.



CONCLUSIONS

The comparative between the three inocula studied, showed how the **granular sludge** coming from an UASB reactor treating brewery wastewaters (sludge III) and the **flocculent sludge** coming from the AD of sewage sludge from a conventional urban wastewater treatment plant (sludge II) gave very similar results in terms of methane production. Moreover, the **flocculent sludge had a higher specific rate constant**. The **microbiologic analysis indicated the importance in selecting an adequate inoculum** for the optimization of AD of SuOC as a result of the resilience of the major components of the bacterial and archaeal communities present in the initial inocula composition.

