Feasibility of sunflower oil cake degradation with three different anaerobic consortia

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ABSTRACT

Sunflower oil cake (SuOC) is the solid by-product from the sunflower oil extraction process and an important pollutant waste because of its high organic content. For the anaerobic digestion of SuOC three different industrial reactors were compared as inoculum sources. This was done using a biochemical methane production (BMP) test. Inoculum I was a granular biomass from an industrial reactor treating soft-drink wastewaters. Inoculum II was a flocculent biomass from a full-scale reactor treating biosolids generated in an urban wastewater treatment plant. Inoculum III was a granular biomass from an industrial reactor treating brewery wastes.

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The highest kinetic constant for methane production was achieved using inoculum II. The inoculum sources were analyzed through PCR amplification of 16S rRNA genes and fingerprinting before (t=0) and after the BMP test (t=12 days). No significant differences were found in the bacterial community fingerprints between the beginning and the end of the experiments. The bacterial and archaeal communities of inoculum II were further analyzed. The main bacteria found in this inoculum belongs to Alphaproteobacteria and Chloroflexi. Of the Archaea detected, Methanomicrobiales and Methanosarcinales made up practically the whole archaeal community. The results showed the importance of selecting an appropriate inoculum in short term processes due to the fact that the major microbial constituents in the initial consortia remained stable throughout anaerobic digestion.

Keywords: Sunflower oil cake, biochemical methane potential, microbial community, fingerprints, methane yield, kinetics.

INTRODUCTION

Sunflower oil cake (SuOC) is the solid waste generated during the sunflower seed oil extraction process. World sunflower seed production ranged between 29.1 and 31.1 million tonnes over the last few seasons. [1] As a result, large quantities of SuOC are generated every year. In Spain alone, between 4 and 5 million tonnes of this by-product are produced, giving rise to an important environmental issue. [2] Current perspectives on how to obtain high value products from wastes involve anaerobic digestion processes for biogas generation [(a mixture of methane and carbon dioxide with a high energetic value (21.4 MJ per m^3)]. These anaerobic processes are performed by

complex groups of microorganisms (Bacteria and Archaea) which coordinate the degradation of organic matter. A relatively low percentage of these microorganisms present in anaerobic digestion processes have been isolated. This lack of knowledge results sometimes in malfunctions and unexplainable failures of biogas fermenters. For these reasons, it must be analyzed in more detail. [3] Only a few studies have considered the potential influence of inoculum in anaerobic digestion systems. Moreno-Andrade and Buitrón [4] studied the influence of five different inocula on an anaerobic biodegradability test of two different substrates, one easily degradable (glucose) and the other toxic (phenol). These authors emphasized the importance of using the appropriate inoculum to obtain satisfactory results from anaerobic processes. After testing two different inocula, granular and suspended, Pereira et al. [5] found granular inoculum to be the best option for the anaerobic treatment of synthetic oleic acid-based effluent, since the methanogenic activity of the granular inoculum was 2-7 times higher than that of the suspended biomass and was more resistant to long chain fatty acid toxicity. Foster-Carneiro et al. [6] compared six different inoculum sources for the anaerobic thermophilic digestion of the organic fraction of municipal solid wastes. Tabatabaei et al. [7] studied the importance of the microbial community, focusing on the methanogenic archaea in the anaerobic digestion of brewery wastewater, palm oil mill effluents, dairy wastes, cheese whey, dairy wastewater, pulp and paper wastewaters and olive oil mill wastewaters with respect to their dominant methanogenic population.

During the process of anaerobic digestion it is expected that the microbial communities adapt as a consequence of the growth of microorganisms under the specific conditions of digestion and the substrate treated. The dynamics of the acetoclastic methanogenic community have been evaluated under the influence of different wastewater compositions and even under inhibitory conditions. [8, 9,

[10] The microbial community structure has been studied under low temperature conditions and under the influence of metal supplementation. [11, 12, 13] However, the transformations which occur in the microbial communities during the anaerobic digestion of organic wastes and methane production are still not fully understood.

It is clear that the efficiency of biogas production during the anaerobic digestion of organic residues depends on the microorganisms involved in the process. The study of these microbial communities represents an important step towards understanding and optimizing these anaerobic treatments. Thus, the aim of this work was to study the influence of the inoculum type on the anaerobic digestion of SuOC in terms of methane production. Microbial community fingerprints from the initial inoculum source and after the biochemical methane potential test (BMP) were compared, determining the major components of the communities involved in the process to achieve the best methane production kinetics.

MATERIALS AND METHODS

Substrate

The substrate used in this study was SuOC. Prior to the experiments, a study of the different particle sizes present in this solid waste was carried out by separation with a mechanical sieve. The most abundant size found (29.4%) was 0.7-1.0 mm. Consequently, this size was used in the experiments. Table 1 shows the full composition and main features of the SuOC used in this study (mean values are averages of four determinations).

Inocula

Three different inoculum sources were used: a) an anaerobic granular inoculum derived from a full-scale upflow anaerobic sludge blanket (UASB) reactor treating wastewaters from a soft-drinks industry (I); b) a flocculent anaerobic inoculum from a full-scale completely stirred tank reactor (CSTR) treating biosolids from a conventional urban wastewater treatment plant (II); and c) an anaerobic granular inoculum from a UASB reactor treating brewery wastes (III). Table 2 shows the main characteristics of these three inocula. The experiments were carried out at an inoculum:substrate ratio of 2:1. An inoculum concentration of 15 g VS L^{-1} was used for each reactor.

Reactors and Operational Conditions

The experiments were carried out in a thermostatized water bath (35ºC) in batch mode. The reactors were stirred at 250 rpm with a magnetic stirrer. The BMP test was run by triplicate. Two controls without substrate were added in each run. A final working volume of 250 mL was used for each treatment. Methane production was measured by a NaOH solution (3N) displacement (CO_2 produced in the anaerobic process was kept in this sodium hydroxide solution).

Experimental Set up

The experiment was carried out by triplicate and two control reactors with no substrate added were run for each different inoculum. The reactors were filled with 15 g VS L^{-1} of inoculum, the corresponding quantity of SuOC to reach a ratio of 2:1 inoculum to substrate, 25 mL of a 50 g $NaHCO_3$ L^{-1} solution to keep pH stable, 50 mL of nutrient solution (Table 3) and distilled water to a total volume of 250 mL. Methane production was measured for a period of 12 consecutive days.

Analytical Methods

Solids and moisture were determined according to the standard methods 2540B and 2540E. [14] Total chemical oxygen demand was determined using the solid substrate open reflux method. [15] Total protein was determined by multiplying the total Kjeldahl nitrogen (TKN) value by 6.25.[16] Fat content was extracted by a soxhlet system using hexane (UNE-EN-ISO 659:2000). Cellulose, hemicellulose and lignin were determined by the Goering and Van Soest method. [17] The elemental composition of the SuOC (C, N, O and H) was measured using a Leco CHNS-932 (Leco Corporation, St Joseph, MI, EEUU) elemental analyzer. For particle size selection the sunflower oil cake was sieved using a mechanical sieve (bio-meta, Retsch).

Methane Production Kinetics

A first-order kinetic model was used to estimate the specific rate constant according to Chen-Hashimoto Equation 1: [18]

$$B=B_o\,[1-\exp(-k\,t)] \qquad (1)$$

where: B is the methane yield (mL CH_4 g^{-1} VS added), B_o is the ultimate or maximum methane yield, asymptote to the production curve *versus* time, k (day^{-1}) is the specific rate constant, and t is the digestion time (days). Methane yield values (B) were calculated by subtracting methane produced by the controls (inoculum only) from their corresponding treatment reactors. These differences were divided by the VS of the substrate. [18] B_o and k were calculated from the experimental data by non linear regression using Sigmaplot 9.0 (Systat Software. Inc., San Jose, CA).

Molecular Characterization of Microbial Communities

Microbial communities, both *Archaea* and *Bacteria*, 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 (Macherey-Nagel, Düren, Germany) according to the manufacturer's recommendations. Fragments of the 16S ribosomal RNA (16S rRNA) genes from the Bacteria and Archaea were amplified by PCR with different primer pairs. Fingerprints of the bacterial and archaeal communities were obtained by Denaturing Gradient Gel Electrophoresis (DGGE) following the method described by Muyzer et al. [19] DNA was directly amplified by PCR using the primer pair 341F-GC (5'-CCT ACG GGA GGC AGC AG with a GC-rich tail attached to its 5' end) [19] and 518R for the Bacteria and the primer pair 344F-GC (5'- with a GC-rich tail attached to its 5' end) and 518R for the Archaea. Relative quantification of molecular fingerprints from pairs of community profiles was performed following the quantitative procedure described by Portillo and Gonzalez. [20] Gels obtained by DGGE were digitalized using Kodak 1D image analysis software

(Kodak, New Haven, CT). The images were analyzed using the tnimage program (http://entropy.brneurosci.org/tnimage.html) applying its densitometry function. Comparisons between community fingerprints were carried out as described by Portillo and Gonzalez [20] calculating a Cramér-von Mises-type statistic through a Monte-Carlo test procedure to determine the significance of differences between microbial communities.

PCR products for 16S rRNA gene library construction were obtained with the primer pair 27F (5'-AGA GTT TGA TYM TGG CTC) and 907R (5'-CCC CGT CAA TTC ATT TGA GTT T) for the Bacteria [21] and the pair 20bF (5'-YTC CSG TTG ATC CYG CSR GA) and 1492bR (5'-GGY TAC CTT GTK WCG ACT T) for the Archaea. [22] These PCR products were purified with the PCR purification kit (JetQuick, Germany) and cloned using a TOPO-TA cloning kit (Invitrogen, Carlsbad, USA). The 16S rRNA libraries obtained were used to identify the major components of the bacterial and archaeal communities. A screening procedure based on the discrimination of clones using PCR-DGGE previously described by Gonzalez et al. [23] was applied to these libraries to identify the major DNA bands observed in DGGE analyses.

Sequence data were edited using Chromas software, version 1.45 (Technelysium, Tewantin, Australia). Homology searches from the nucleic acid sequences were performed using the Blast algorithm [24] at the NCBI (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/Blast/). Sequences were inspected for the presence of chimeras using the Ccode program as described by Gonzalez et al. [25]

RESULTS AND DISCUSSION

The volumes of methane (at standard temperature and pressure) obtained after 12 days of the BMP test for inocula II and III were higher than that obtained for inoculum I (293, 360 and 387 mL CH_4 for inocula I, II and III, respectively). Methane production for inoculum III was 7.5% higher than for inoculum II and 31.1% higher than for inoculum I. The experimental methane yields per gram of VS added (*B*) are shown in Figure 1. The best *B* values after 12 days were obtained for inocula II and III (193 and 205 mL CH_4 accumulated g^{-1} VS added, respectively), these yields being higher than that obtained for inoculum I (156 mL CH_4 accumulated g^{-1} VS added). The value of the methane yield for inoculum III was 6.2% higher than for inoculum II, which in turn was 23.7% higher than the value for inoculum I. The yield for inoculum III was 31.4% higher than for inoculum I. Therefore, inocula II and III had similar methane yields and were both higher than for inoculum I.

The percentage of volatile solids removed was 42% for inocula II and III and only 33% for inoculum I. Inocula II and III from industrial reactors treating solid substrates showed better results than inoculum I from wastewater treatment. This could be attributed to the higher hydrolytic/enzymatic capacity of these inoculum sources which are used to break biosolids in urban wastewater treatment plants (inoculum II) and to treat brewery wastes (inoculum III).

The cellulose, lignin and hemicellulose structure of SuOC is complex. Cellulose is a polymer with low microbial degradability and is considered the rate-limiting substrate in the anaerobic digestion of solid wastes. [26] In a comparative study for cellulose solubilisation in anaerobic reactors, O'Sullivan et al [27] showed how differences in reactor configuration and operational conditions had no significant impact on the solubilisation rate of cellulose, whereas the difference in composition

206 of the microbial communities showed a marked effect. This could be the reason why inoculum I,
207 which had thus far been used to treat wastewaters, had given the worst results as regards methane
208 production and kinetics for SuOC treatment. These findings should be studied in more detail.
209 The first-order kinetic model used to estimate the specific rate constants fit satisfactorily to the
210 obtained experimental data (with R^2 values higher that 0.965; Fig. 1). The values obtained for *k*
211 were 0.11±0.02, 0.37±0.01 and 0.34±0.01 $days^{-1}$ for inocula I, II and III, respectively (Table 4).
212 Therefore, the specific rate constant for inoculum II was 8.8% higher than that achieved for
213 inoculum III and 236.4% higher than that obtained for inoculum I.
214
215 Figures 2 and 3 show the molecular fingerprints obtained by PCR-DGGE and represent the major
216 components of the bacterial (Fig. 2) and archaeal (Fig. 3) communities from the different inoculum
217 sources (I, II and III) used during this study. For inoculum II, the taxonomic affiliation and the
218 accession numbers of the closest homologue for the major components of the bacterial and archaeal
219 communities are given in Tables 5 and 6, respectively. Comparisons of fingerprints from the
220 bacterial and archaeal communities for the three inoculum sources used in this study (Figs. 2 [A, C
221 and E] and 3 [G, I and K]) showed distinctive banding patterns which would indicate distinct
222 microbial communities among the three inocula, depending on their source.
223
224 Maximum methane production was reached after nine days for inocula II and III and after twelve
225 days for inoculum I. After 12 days' digestion time, the bacterial communities (Fig. 2 [B, D and F])
226 established in the anaerobic digestion process of the SuOC, showed similar fingerprinting profiles
227 to those of the bacterial communities in their respective inocula (Fig. 2 [A, C and E]) before the
228 anaerobic process. Statistical comparison of fingerprints from the initially inoculated communities

and the final communities after the BMP test showed no significant differences (Table 7) in the bacterial communities from the different inoculum sources used in this study.

After the anaerobic digestion process of sunflower oil cake (Table 7), no significant differences were found in the archaeal community fingerprints between the initial inoculum (Fig. 3 [I and K]) and inocula II and III (Fig. 3 [J and L]). However, significant differences were observed between the initial inoculum (Fig. 3 [G]) and the archaeal community developed (Fig. 3 [H]) in inoculum I. Despite this change in the structure of the archaeal communities in inoculum I, the major archaeal components remained as important members of the final (after the anaerobic digestion process) communities. Changes observed in specific archaeal phylotypes in inoculum I could be the cause of a reduced performance of the process when compared to the evolution of inocula II and III which were maintained during anaerobic digestion.

The bacterial and archaeal communities from inoculum II where the inoculum showed optimum methane kinetic parameters, was studied in further detail to identify the major components of the communities implicated in the anaerobic digestion and methane production. Table 5 shows the proportion of the major bacterial constituents of the community in inoculum II. Alphaproteobacteria (20.6% and 28.8% of the total identified DNA in the inoculum and after anaerobic digestion, respectively), within the Rhodobacteraceae Family (e.g., *Paracoccus*), and Chloroflexi (22.6% and 23.4% of the total bacteria in the inoculum and in the community developed after anaerobic treatment, respectively) were the dominant bacterial groups. 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*), Actinobacteria (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).

The major bacterial components constituting the community of the anaerobic digestion process of sunflower oil cake coincide with the bacterial groups present in communities reported for other wastes. [22, 28] Proteobacteria, Chloroflexi and Firmicutes have been reported as major components in bacterial communities during the anaerobic digestion processes of organic wastes. [22, 29, 30] Chloroflexi has recently been shown as a highly significant component in the transformation of complex substrates such as olive residues from oil production and this bacterial phylum is being increasingly recognized for its importance in anaerobic systems. [22, 29-31] In these communities, numerous phyla which are not well-known, such as the Bacteroidetes, Synergistetes and the Candidate Division WS6, were detected. At present, there is limited knowledge about the metabolism of these phyla and they are generally detected only by their 16S rRNA gene sequences. Furthermore, there is little or no availability of representative cultivated microorganisms belonging to these bacterial phyla, which indicates that there is a significant portion of the bacterial community in need of further physiological research. The importance of Synergistetes, for instance, in anaerobic treatments has been highlighted in recent studies [32-33], as has the presence of Candidate Division WS6 in anaerobic waste treatments and its relationship to methanogenic Archaea. [34]

Archaea are the microorganisms responsible for the production of methane. The archaeal communities represented by methanogenic groups constituted a critical component of the prokaryotic communities leading to methane production. Table 6 shows the proportion of the major archaeal phylotypes in inoculum II. The detected sequences from the archaeal community all corresponded to methane-producing Archaea. Different archaeal phylotypes were detected in the anaerobic digestion process of sunflower oil cake and belonged to the Methanosarcinales and Methanomicrobiales orders. The Methanosarcinales, mainly represented by different phylotypes belonging to the genus *Methanosaeta*, were the dominant methanogens, constituting over 67% of the archaeal community.

A dominance of the methanogens Methanosarcinales and Methanomicrobiales has been previously reported as indicators of well-established methane-producing anaerobic digestion processes. [22, 35, 36] These methanogens are acetoclastic methane producers and confirm the importance of this pathway in methanogenesis, as seen during the digestion of SuOC. As a consequence, a direct interaction between bacteria and archaea is envisioned, the main role of the bacterial community during this anaerobic process appeared to be the production of acetate from the polymers constituting the SuOC. This acetate is the major substrate which is directly utilized by the methanogenic archaea as the source for methane production.

CONCLUSIONS

The results obtained during this study underline the importance of using productive and active inoculum sources to initiate anaerobic digestion processes of sunflower oil cake wastes. Microbial

communities showed no changes during short term experiments (12 days). Obtaining the highest possible SuOC treatment efficiencies is a consequence of the conservation of the major components of well-established bacterial and archaeal communities during the digestion treatments. Only when an optimal inoculum is used can methane production and degradation of the processed substrate (i.e., SuOC) be maximized. A loss or reduction in specific phylotypes during the anaerobic treatments can be reflected by a diminishing efficiency both in methane production and organic load degradation.

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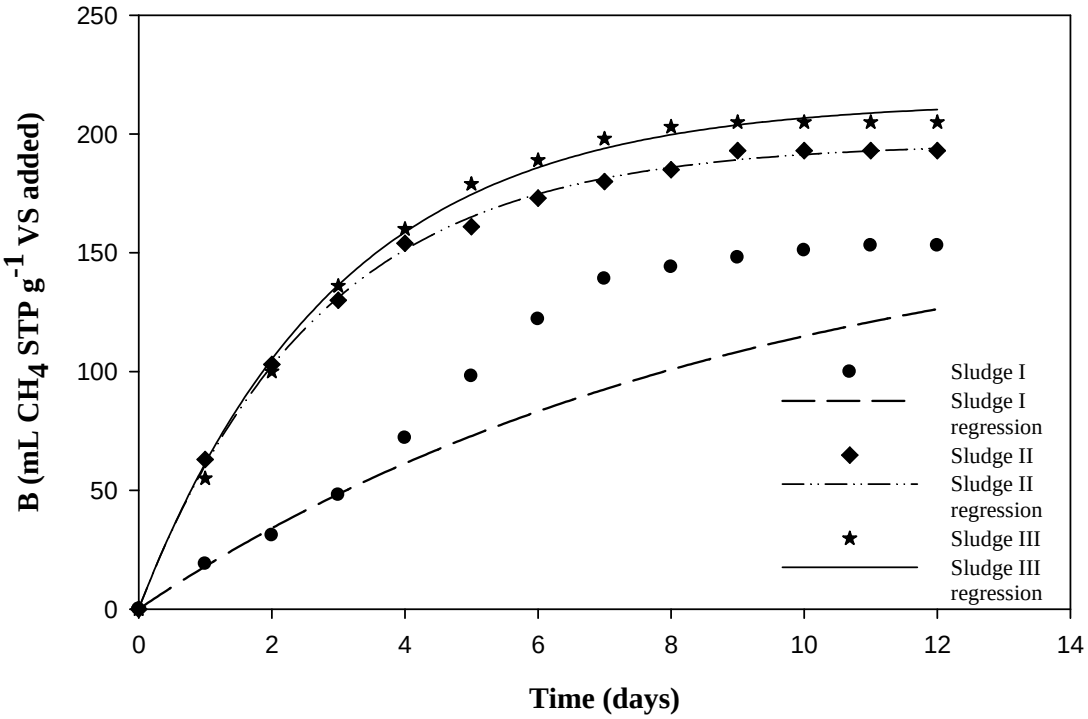
FIGURE CAPTIONS

Figure 1. Variation of the volume of methane produced per gram of VS added over time for inocula I, II and III.

Figure 2. Bacterial community fingerprints obtained by PCR-DGGE: (A, C, E) for the three different inoculum sources used for the initial inoculation of reactors and (B, D, F) after the BMP tests at the end of the anaerobic SuOC treatments.

Figure 3. Archaeal community fingerprints obtained by PCR-DGGE: (G, I, K) for the three different inoculum sources used for the initial inoculation of reactors and (H, J, L) after the BMP tests at the end of the anaerobic SuOC treatments.


250
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B (mL CH4 STP g-1 VS added)
Time (days)
Sludge I
Sludge I regression
Sludge II
Sludge II regression
Sludge III
Sludge III regression

Fig. 1

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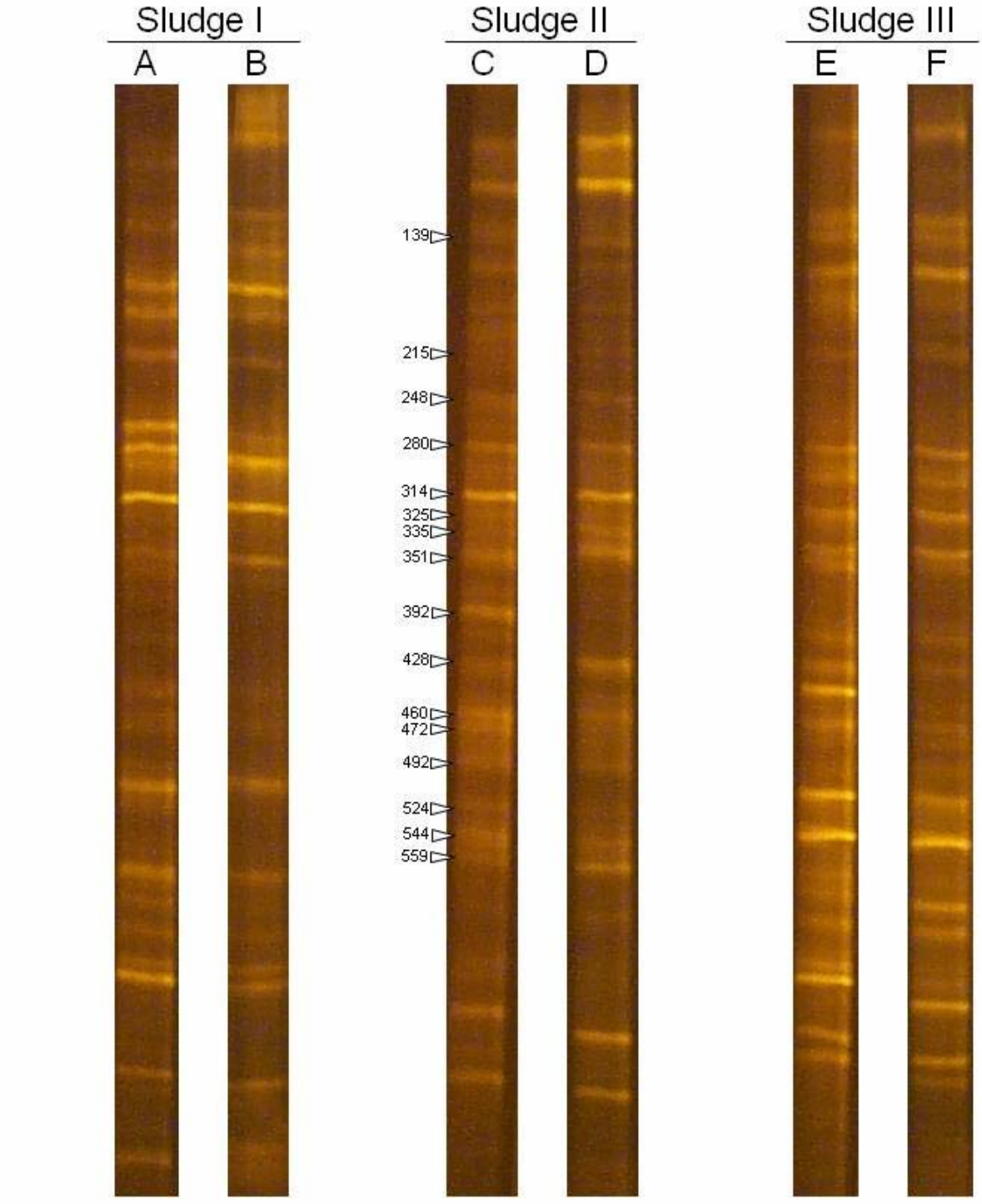

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470 Fig. 2

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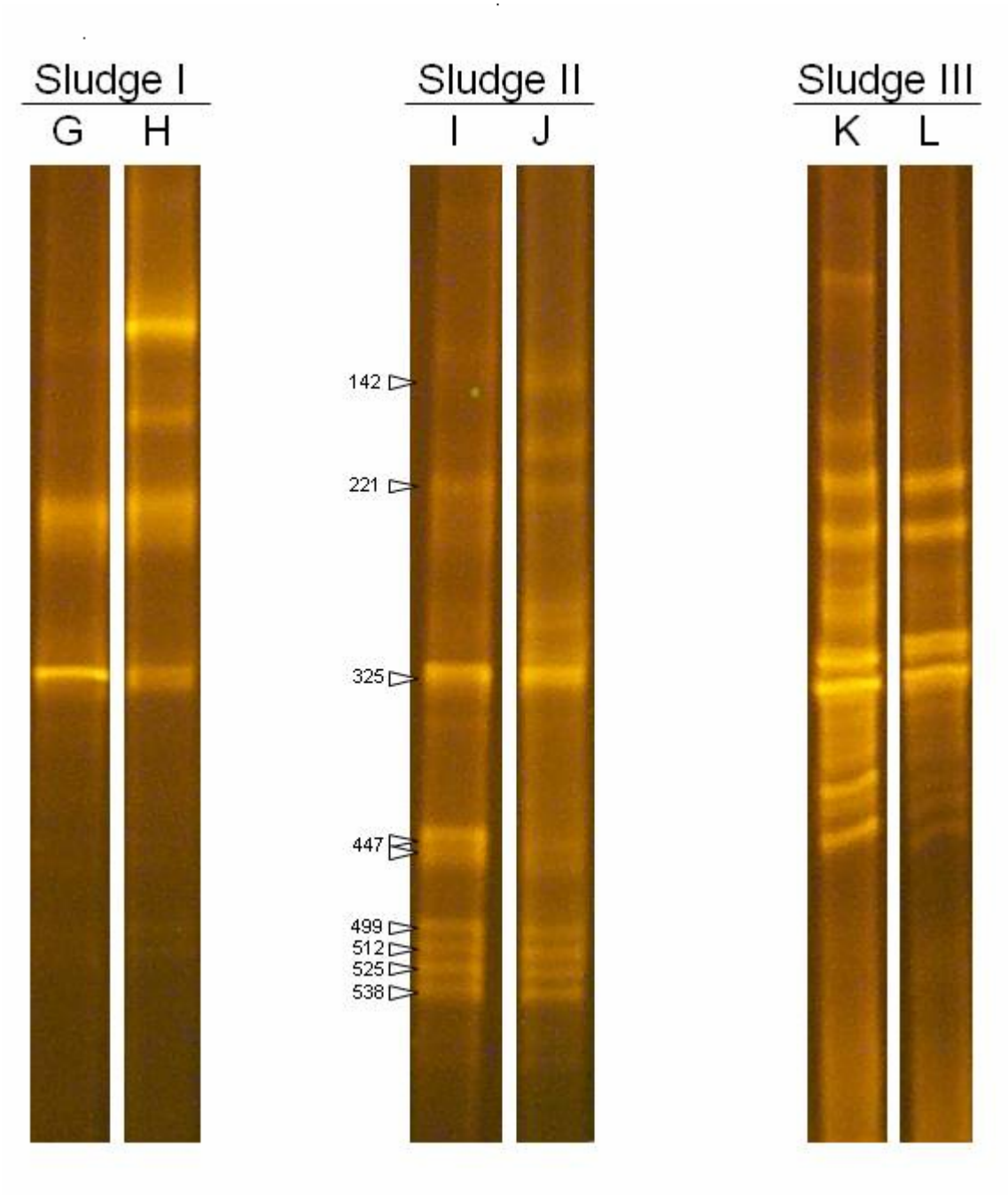

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474 Fig. 3

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Table 1. Characteristics of the SuOC used as substrate.

Parameter*	Value±SD**
Moisture (%)	8.0±0.5
Total protein (%)	31.4±1.6
Fats (%)	1.7±0.1
Carbohydrates (%)	58.7±2.6
Hemicellulose (%)	9.2±0.5
Lignin (%)	9.5±0.4
Cellulose (%)	21.7±1.1
TS (%)	93.4±1.9
MS (%)	6.6±0.1
VS (%)	86.5±1.3
TCOD (g O_2 g^{-1} TS dry basis)	1.08±0.04
C (%)	43.6± 0.3
H (%)	6.2± 0.1
N (%)	4.6± 0.6
O (%)	45.6± 0.5

*TS: total solids, MS: mineral solids, VS: volatile solids, TCOD: total chemical oxygen demand. **SD: standard deviation.

Table 2. Characteristics and origin of the inoculum sources used in the experiments.

Sludge	Source (Reactor type)	Reactor volume (m^3)	pH	TS ($g\ L^{-1}$)	VS ($g\ L^{-1}$)
I	UASB	450	7.4	30	25
II	CSTR	2000	7.6	43	20
III	UASB	550	7.5	83	47

TS: total solids; VS: volatile solids; UASB: upflow anaerobic sludge blanket; CSTR: continuously stirred tank reactor.

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507 **Table 3.** Composition of the nutrient and trace element solutions used.

Nutrient solution composition	Concentration ($g\ L^{-1}$)
NH_4Cl	1.4
K_2HPO_4	1.25
$MgSO_4\ H_2O$	0.5
$CaCl_2\ 2H_2O$	0.05
Yeast extract	0.5
Trace element solution	5.0[a]
Trace element solution composition	**Concentration ($mg\ L^{-1}$)**
$FeCl_3 4H_2O$	2000
$CoCl_2 \cdot 6H_2O$	2000
$MnCl_2\ 4H_2O$	500
$CuCl_2\ 2H_2O$	38
$ZnCl_2$	50
H_3BO_3	50
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	50
$AlCl_3\ 6H_2O$	90

Units for the trace element solution added to the nutrient solution are in mL of trace solution per L of nutrient solution ($mL\ L^{-1}$).

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Table 4. Values of B_o and k obtained using the Chen-Hashimoto equation for the three sludges studied and their variation coefficients.

Sludge	R^2	B_0 ± SD (mL CH_4 g^{-1} SV added)	k ± SD ($days^{-1}$)	VC_{B0} (%)	VC_k (%)
I	0.9648	172 ± 27	0.11 ± 0.02	15.5%	25.4%
II	0.9985	196 ± 1	0.37 ± 0.01	0.6%	2.1%
III	0.9964	214 ± 2	0.34 ± 0.01	1.1%	3.6%

SD: standard deviation; VC: variation coefficient

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535 **Table 5**. Accession numbers of closest homologue and proportions of the major bacterial
536 phylotypes identified during this study determined through community fingerprinting analysis using
537 PCR-DGGE from inoculum II.

Migration	Taxonomic affiliation (Accession No. of closest homologue)	Fraction inoculum*	Fraction BMP*
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	*Thauera*, Betaproteobacteria (DQ098974)	5.6	1.0
428	Bacteroidetes (CU922674)	2.7	6.1
460	*Paracoccus*, Alphaproteobacteria (FJ386516)	5.7	4.8
472	Chromatiales, Gammaproteobacteria (AM176837)	4.4	1.5
492	Thermoanaerobacteriales, Firmicutes (EU878332)	2.1	2.5
524	*Synergistes*, 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

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

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Table 6. Accession numbers of closest homologue and proportions of the major archaeal phylotypes identified during this study determined through community fingerprinting analysis using PCR-DGGE from inoculum II.

Migration	Taxonomic affiliation (Accession No. of closest homologue)	Fraction inoculum*	Fraction BMP*
142	Methanosarcinales (FJ705109)	6.0	7.7
221	*Methanosaeta*, Methanosacinales (AB494241)	12.1	7.0
325	*Methanosaeta*, Methanosarcinales (FM162203)	20.5	28.8
447	Methanosarcinales (GU196156)	16.9	11.4
499	*Methanosaeta*, 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

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

Table 7. Statistical results of the comparison between the microbial communities at the beginning (inocula) and ending of the anaerobic treatment of sunflower oil cake for the three types of inoculated sludges.

Inoculated sludge	Archaea		Bacteria	
	P	CV (%)	P	CV (%)
I	0.023*	0.098	0.170	0.093
II	0.188	0.081	0.211	0.079
III	0.542	0.046	0.316	0.068

P: Probability values; CV: coefficient of variation. Asterisk indicates significant differences ($P<0.05$).