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TGGE-MONITORING OF THE MICROBIAL COMMUNITY ALONG THE OLIVE MILL WASTEWATERS ANAEROBIC TREATMENT

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ABSTRACT
Monitoring the microbial community present during the olive mill wastewater (OMW) anaerobic treatment was carried out using PCR-TGGE analysis. Phase-contrast microscopy was used in order to directly examine microbial cells morphology. Samples were collected from the anaerobic digester bottom along the successive increases of OMW loading rate. TGGE banding patterns showed a significant diversity of OTUs. Cluster analysis of TGGE banding patterns shows two major groups: one cluster composed of samples belonging to the initial phases of treatment and a second cluster that encloses the other two samples. According to BLAST results, four sequences affiliated with group *Cytophaga-Flexibacter-Bacteroidetes* (CFB), one with sub-class Epsilon-Proteobacteria and other with phylum Firmicutes. The sequences obtained from each band were used to construct phylogenetic trees, and also using bacterial 16S rDNA sequences from environmental clones and reference taxa. Archaeal 16SrDNA PCR detection analysis revealed the presence of Archaea only in the initial sample of the digester start-up suggesting that methanogenesis along the treatment process occurs at an upper level of the unit. Concerning microscopic observations, the more representative morphological forms found in the bottom of digester were *Clostridium* spp. and *Clostridium* spp. sarcina-like forms.

Keywords: molecular monitoring, microbial dynamics, anaerobic digestion, biogas, olive mill wastewater

INTRODUCTION
Molecular monitoring provides information on the composition of any microbial consortium allowing a detailed characterization of the entire community, including the microorganisms present in a low percentage, as well as the non-culturable microorganisms. This advantage is even more emphasized when dealing with microorganisms from extreme and/or highly sensitive habitats and/or for the management of microbial communities to provide services to society e.g. effluent bio-treatments.
Biological processes play a major role in the overall effluents treatment efficiency, taking profit of the effluents native microbial catabolic diversity to degrade, transform or accumulate an extended range of compounds and also their natural adaptation to those specific environments. For this, it is of major relevance to evaluate the potential and understand the role of the indigenous microbial populations, and also to monitor the microbial community developed along a treatment process. Moreover, optimal treatment conditions can be established for the target population and thus favour and improve the treatment.
The use of molecular methods based on 16S rRNA genes are widely used in combination with techniques such as TGGE/DGGE (Thermal Gradient Gel Electrophoresis/Denaturing Gradient Gel Electrophoresis) to characterize microbial communities from a wide variety of samples [1-4]. These fingerprinting techniques enable the analysis of multiple samples simultaneously, the assessment of microbial communities structure and the determination of their dynamical responses to environmental perturbations.

Olive mill wastewaters (OMW), carrying a high organic content, is a potential substrate for biogas production. However, OMW is a problematic effluent and unsuitable for direct anaerobic treatment. This is due to the high organic content, presence of toxic/inhibiting substances (lipidic and phenolic compounds), unfavourable C/N ratio, low ammonia levels and alkalinity and to the acid pH values. High dilutions, addition of alkalis and nitrogen source and physicochemical and biological pre-treatments are performed in order to mitigate the OMW concentration and inhibiting capacity [5-7].
Different operational procedures are tested at INETI-DER and a successful methodology is achieved to recover the energetic contents of OMW without any previous alteration of the substrate [8-10]. In order to characterize and analyze the microbial community structure and to infer its dynamics during the anaerobic digestion process, a fingerprinting study was carried out based in 16S rDNA PCR-TGGE analysis. For this purpose, four final stages of the anaerobic process were selected, in which the highest concentration of OMW was digested. In addition, phase-contrast microscopy was used for direct cell counting and to morphology observations.

MATERIAL AND METHODS

Anaerobic digester

A laboratorial up-flow anaerobic filter of 2.5 L total volume is used to digest continuously different OMW concentrations under mesophilic temperature conditions. It is seeded with bio-solids from an industrial anaerobic digester plant in *Santarém (Portugal*) and operated by increasing the OMW loading rate into the digester feed. OMW are obtained from a continuous three phases olive mill also in *Santarém* region.

Sample collection

Four samples were collected from the bottom of the anaerobic digester in the final phases of anaerobic process that were designated by DER.1, DER.2, DER.3 and DER.4 (166, 206, 252 and 297 days of treatment, respectively). They were collected from the unit when the operating conditions were amended by increasing the OMW loading rate. The sample of the biosolids used at the start-up of the digester operation was nominated by DER.0. All samples were stored at 4º C in sterile containers until further use.

DNA extraction and PCR amplification

For each collected sample, total DNA was extracted using the UltraClean™ Soil DNA Kit (MoBio Laboratories, Inc.), following the manufacturer's instructions. DNA samples were stored in TE buffer at -20ºC until further use. All PCR reactions were performed in 50 μl reaction mixtures containing 1x PCR buffer (PCR buffer without $MgCl_2$: PCR buffer with $(NH_4)_2SO_4$, 1:1), 3 mM $MgCl_2$, 5 % dimethylsulfoxide, 200 μM each nucleotide, 0.3 μM each primer, 1 U Taq polymerase and 50-100 ng purified template DNA. For 16S-PCR-TGGE, it was amplified the V3 region of bacterial 16S rDNA using 341F and 534R as PCR primers. A GC clamp was attached to the 5' end of the forward primer to be used in a TGGE system [4]. The amplification cycles were as follows: initial denaturation at 94ºC for 5 min; 35 cycles of denaturation at 94 ºC for 1 min, annealing at 50ºC for 1 min, and extension at 72 ºC for 1 min. The final extension was at 72ºC for 30 min. Archaeal 16S rDNA was amplified using 344F and 934R [1] as PCR primers. Amplification cycles were performed as described for bacterial 16S rDNA excluding annealing step performed at 53º C. The PCR products were analyzed by electrophoresis in 1% agarose gel in 0.5x TAE buffer, stained by ethidium bromide (5 mg.l-1), visualized under UV light and documented using Molecular Imager FX™ System (BioRad, Richmond, USA).

TGGE analysis

The TGGE analysis was performed using a TGGE Maxi System (Biometra). PCR-TGGE amplicons were loaded in polyacrylamid gels (6% [wt/vol] acrylamid, 7M urea and 2% glycerol, in 0.1X TBE buffer). The gels were electrophoresed in a thermal gradient from 41ºC to 49ºC, at a constant voltage of 150 V for 16h, and stained following the silver staining protocol. Gel images were registered. For TGGE profiles analysis, each band was described by its position and each sample (gel lane) was compared to all gel lanes. Cluster Analysis was done using unweighted pair group

method with mathematical averages (UPGMA). Correlations were calculated using the Dice coefficient of similarity. A relatedness tree was produced with the algorithm of the NTSYSpc2 software.

DNA sequencing and affiliation studies

TGGE bands that were apparently correlated to dominant members were selected for excision from TGGE gels, placed into sterilized vials containing 30 µl of sterilized distilled water and stored overnight at 4°C to allow the DNA to passively diffuse out of the gel strips. Ten microlitres of eluted DNA was used as the template for amplification with the primers 341F_GC and 534R, as described above. Running the collected band and the original sample side by side in a new TGGE gel checked the accuracy of the process. Whenever necessary, bands were re-excised and treated as described above. For sequencing analysis, PCR products were purified with the Concert™ Rapid PCR Purification System (Gibco BRL, Eggenstein, Germany) and used as template in the sequencing reactions. To determine the phylogenetic affiliation, similarity search was performed using the BLAST program [11]. The nucleotide sequences were aligned by the CLUSTAL X program and the phylogenetic tree was constructed [12].

Phase-contrast microscopy

Evolution of bacterial community along the anaerobic process reactor was observed and registered by phase-contrast microscopy, Olympus BX51, with an oil-immersion objective. The number of bacteria (cell mL^{-1}) in different samples from the anaerobic reactor along the study was determined microscopically by direct count with a Thoma counting chamber. At least 16 microscopic fields were monitored from each count.

RESULTS AND DISCUSSION

Phylogenetic affiliation studies

TGGE analysis of PCR-amplified genes coding for 16S rRNA was used to assess the structure and dynamics of bacterial community of all samples (Fig. 1B). Banding patterns on TGGE gel with primers 341F_GC and 534R showed good resolution and separation in a 41 - 49ºC thermal gradient as well as a significant diversity of OTUs (Operational Taxonomic Units). Furthermore, common bands identified at the same relative position in different lanes revealed variable relative intensities, which might indicate a difference in the relative abundance of certain common bacterial species present along the treatment period. Cluster analysis was performed using UPGMA and correlations were calculated using the Dice coefficient of similarity. A dendrogram was produced with the algorithm of the NTSYSpc2 software (Fig. 1B).

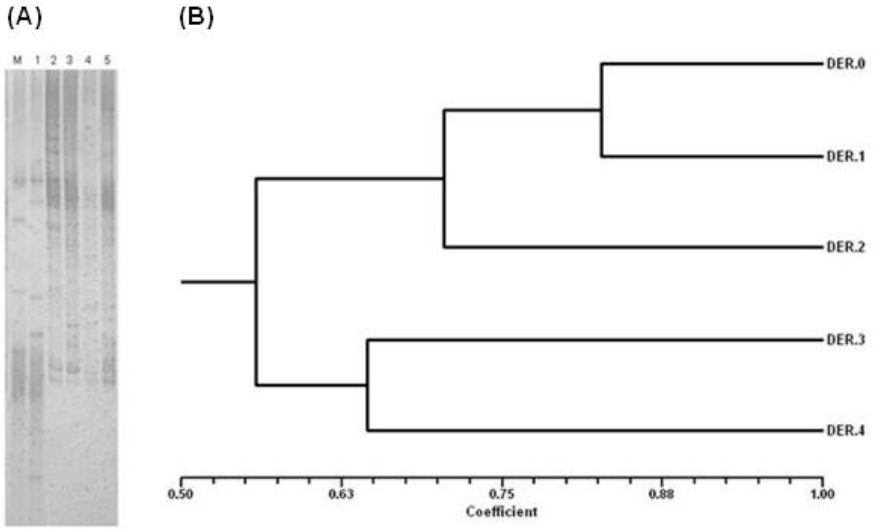

Fig. 1 - (A) TGGE fingerprints of PCR-amplified bacterial 16S r-DNA fragments for the 5 samples (M- TGGE 100 bp marker, 1- DER.0, 2- DER.1, 3- DER.2, 4- DER.3, 5- DER.4), using a 41º to 49ºC thermal gradient. (B) Cluster analysis of bacterial communities based on TGGE profiles. Similarity matrices were calculated with the DICE coefficient and dendrogram was generated using the UPGMA method (r= 0.89).

The total number of band positions detected in the TGGE banding patterns obtained for the 5 samples under study was 31 and the number of TGGE bands per sample varied from 12 to 18. TGGE profile obtained for sample DER.0 presented the lowest diversity (12 OTUs) and DER.2 showed the highest (18 OTUs).
Cluster analysis of TGGE banding patterns shows two major groups: one cluster composed of samples DER.0 to DER.2 (initial phases of treatment) and a second cluster that encloses samples DER.3 and DER.4 (final stage of treatment).
To determine the identity of organisms represented by bands in TGGE profiles, bands were excised from TGGE gels for sequencing. It was possible to obtain six nucleotide sequences. For phylogenetic allocation, each sequence was submitted to a BLAST search. Results of their closest relative are shown in Table 2.

Table 2: Phylogenetic sequence affiliation and similarity to the closest relative of TGGE band DNA sequences

Band no.	Closest Relative (accession no.)	Origin	Similarity	Phylogenetic affiliation
BD1	Uncultured anaerobic bacterium clone 44 st3 0-2cm (EU290711)	Sea sediments (Namibia)	88%	CFB group
BD2	Swine manure pit bacterium PPC87 (AF445250)	Swine manure pit	84%	CFB group (Bacteroidetes)
BD3	Uncultured bacterium isolate DGGE band 40-60%_D7 (DQ054740)	Cows rumen liquid	91%	CFB group (Bacteroidetes)
BD4	Uncultured epsilon proteobacterium clone GoM GC234 613E (AY211676)	Gas hydrate sea sediments (Gulf of Mexico)	94%	epsilon-Proteobacteria
BD5	Uncultured bacterium clone M35_D8_L_B_E10 (EF586006)	solid waste digester fed with methanol	86%	CFB group (Bacteroidetes)
BD6	Uncultured *Clostridium* sp. clone 8-2 (AY883110)	Anaerobic sludge	86%	Firmicutes

According to BLAST results, four sequences affiliated with group *Cytophaga-Flexibacter-Bacteroidetes* (CFB), one with sub-class Epsilon-Proteobacteria and one with phylum Firmicutes.
The sequences obtained from each band were used to construct phylogenetic trees, and also using bacterial 16S rDNA sequences from environmental clones sequences and reference taxa from Epsilon-Proteobacteria, Firmicutes and the CFB– group. *Acidoccus sulfurreducens* was used as outgroup (Fig. 2).

Archaeal 16SrDNA-PCR detection analysis (data not shown) revealed the presence of Archaea only in sample DER.0. Although a good production of methane could be obtained along the anerobic digestion process, indicating an obvious presence of methanogenic bacteria (Archaea domain) and since it was not detected in any of the samples collected at the digester bottom, it is our hypothesis that methanogenesis is occurring at an upper level of the digester. Hence, within the settled biomass, non-methanogenic microorganisms known to be involved in biogas production pathway, namely acidogenic and acetogenic bacteria, were detected.

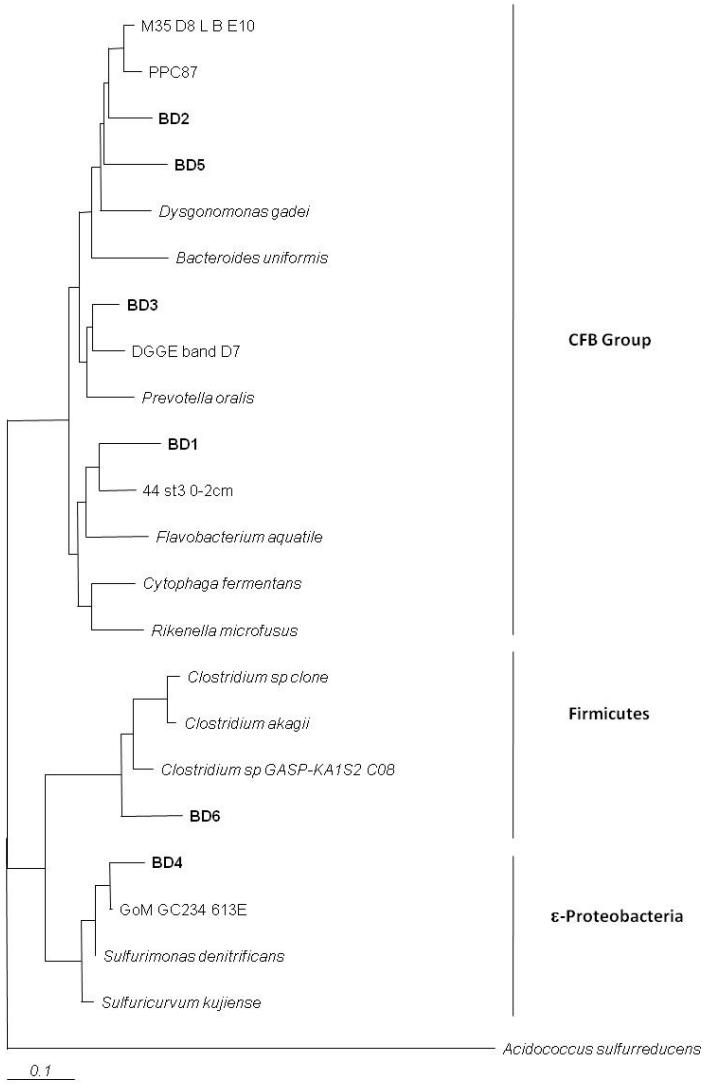

Fig. 2: Phylogenetic relationships between sequences retrieved from TGGE bands during this study (bold), reference taxa and available environmental clones, using *Acidoccus sulfurreducens* as outgroup.

Phase-contrast microscopy analysis

All samples collected during anaerobic process had a mean of 10^9 cells/ml emphasizing the stability of the microbial community in the bottom of digester.

Concerning morphological observations higher diversity seems to be present in samples DER.3 and DER.4 than in the other samples. The more representative morphological forms found were represented in Fig. 3: *Clostridium* spp. (C and D) and *Clostridium* spp. sarcina-like forms (B and E). These morphological observations are in agreement with the molecular results, since by TGGE analysis, *Clostridium* spp. were identified in all samples under study.

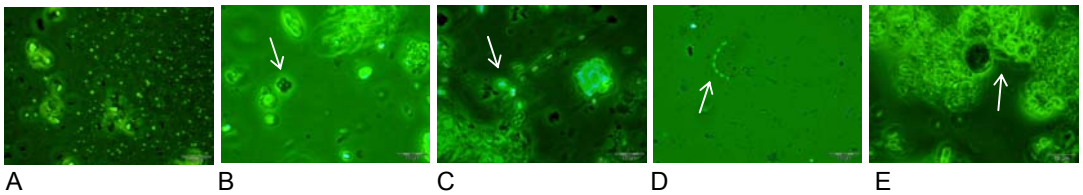

Fig.3 – Phase-contrast micrographs of bacterial community along the anaerobic process: A (DER 0), B-D (DER 3) and E (DER 4).

CONCLUSIONS

This work has shown that molecular methods such as TGGE analysis are adequate to study microbial communities present in an environment such as an anaerobic digester, and constitute valuable tools for complementing and gather information, leading to an improvement of overall treatment. Future work will focus on the characterization and identification of Achaea dominant species occurring throughout the bio-treatment period, especially methanogenic communities that are probably be positioned at the upper section of the digester. Also, FISH (fluorescent *in situ* hybridisation) method will be combined with TGGE analyses to add quantitative data and to gain a more comprehensive spatial and temporal picture of the compositional structure and dynamics of the microbial community present in the anaerobic digester.

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