Microbial community dynamics in the two-stage anaerobic digestion process of two-phase olive mill residue

B. Rincón[1*]; M.C. Portillo[2]; J.M. González[2]; R. Borja[1]

[1] *Instituto de la Grasa (CSIC), Avenida Padre García Tejero 4, 41012 Sevilla, Spain*

[2] *Instituto de Recursos Naturales y Agrobiología (CSIC), Apartado 1052, 41080 Sevilla, Spain.*

*****Corresponding author: B. Rincón (Tel: +34 95 4689654; Fax: +34 954691262; E-mail: brlloren@cica.es)

Running Title: "Microbial evolution in anaerobic digestion of olive mill residue"

ABSTRACT

The microbial communities in a two-stage anaerobic digestion process treating olive mill "solid" residues were studied by molecular identification techniques. The microbial species identification in the hydrolytic-acidogenic step and in the methanogenic step was carried out by polymerase chain reaction amplification of 16S ribosomal RNA genes, denaturing gradient gel electrophoresis, cloning, and sequencing. This study revealed that Firmicutes (from 31.1% to 61.1%, average 42.1%) mainly represented by Clostridiales, and the Chloroflexi (from 29.4% to 53.3%, average 47.35%) were the most abundant species for the hydrolytic-acidogenic reactor. Other microorganisms such as Gamma-Proteobacteria (*Pseudomonas* species as the major representative; from 3.9% to 9.7%; average 5.7%), Actinobacteria (from 1.0% to 10.2%; average 4.6%) and Bacteroidetes (from 1.1% to 3.1%; average 1.7%) were also detected. The methanogenic communities detected in the methanogenic reactor were mainly represented by members of the obligate acetotrophic methanogenic genus *Methanosaeta*. *Methanosaeta* was the crucial Archaea to obtain a high methane yield in the methanogenic stage.

Keywords: *Bacterial and Archaeal populations; biomethanization; olive mill solid waste; poly-phenolic compounds.*

INTRODUCTION

Anaerobic digestion (AD) processes are an attractive alternative for high organic load substrates treatments. AD allows a high degradation of the organic components through the action of different groups of microorganisms (hydrolytics, acetogens and methanogens) producing biogas (mixture of methane (60-70 %) and carbon dioxide (30-40 %)) (Amani et al., 2011) with high calorific value (20833-25000 kJ m^{-3}). A low percentage of the microorganisms present in anaerobic digestion processes have been cultured and isolated. This lack of knowledge might lead to malfunction and unexplainable failure of biogas fermenters. Therefore, the influence of the microbial communities on biogas yield and system stability must be analyzed in more detail (Weiland, 2010). The two-phase olive mill "solid" residue (OMSR) generated in the "ecologic" two-phase centrifugation olive oil manufacturing process is produced in high quantities (0.8 kg OMSR kg^{-1} olives milled). Although named "solid", it has a high water content (60-85 %) because it retains all the water from the olive. The high organic content (total chemical oxygen demand (COD) of 162 g L^{-1} after stone removal) of the OMSR and its high concentration of poly-phenolic compounds (15 g L^{-1}) place this waste as a very phytotoxic waste. The disposal and management of the OMSW constitute important environmental problems (Borja *et al.*, 2006). AD processes are attractive solutions for OMSR treatments.

The microorganisms participating in the AD processes have different levels of sensitivity to the environmental conditions, different growing kinetics and show differences in physiological and nutritional requirements (Anderson *et al.*, 1994). The separation of the process in two independent steps, hydrolytic-acidogenic (H-A) and methanogenic (M), is very convenient in some cases. Most of the studies concerning stage separation are related to inhibition and toxicity (Demirel and Yenigun, 2002). In addition, two-stage processes reduce the risk of accumulating toxic intermediates such as volatile fatty acids that can inhibit the development of methanogenic communities (Veeken and Hamelers, 2000). The inhibition of the anaerobic processes by phenols and poly-phenolic compounds has been studied at different temperatures and substrates. Most of these studies point out the importance of acclimation periods to obtain good results (Chen *et al.*, 2008). Wang et al. (1991) studied the inhibition of acetate-utilizing methanogens by phenols. They found how the rate of methane production decreased progressively with increasing concentration of phenolic compounds. A

noncompetitive inhibition model was found to best fit the experimental data with inhibition constants of 1,000–2,000 mg L^{-1} for hydroxyphenols, and 900 mg L^{-1} for phenol. Bacterial genera like *Desulfotomaculum* and *Clostridium*, and Archaea within the *Methanosaetaceae*, *Methanomicrobiales* and *Methanobacteriaceae,* have been described for the anaerobic transformation of different phenols (Fang *et al.*, 2004; Zhang *et al.*, 2005).

The current studies on the microbial communities involved in the OMSR degradation were carried out in one stage, in an unique reactor with all the microorganisms participating in the process working together (Rincón *et al.*, 2008a). None microbiological study has been carried are out in two stages (H-A and M steps) for the OMSR until now. The aim of this work was to study the microbial communities participating in the two-stage anaerobic digestion process treating OMSR and their evolution. The knowledge of the communities best adapted to treat OMSR and the poly-phenolic compounds degradation in order to avoid toxic inhibitions and to obtain the highest methane production could be an important tool for the optimization of the anaerobic treatment of this waste for future industrial purpose.

These experiments were carried out in the labs of the "Instituto de la Grasa" (CSIC) and "Recursos Naturales" (CSIC) in Sevilla, Spain during 2009.

MATERIALS AND METHODS

Two-phase olive mill solid residue used

The waste used in this study was olive mill "solid" residue collected from a two-phase olive oil factory processing olives of the 'Picual' variety ("Instituto de la Grasa" (CSIC), Seville, Spain). The olives were harvested at the beginning of the olive season with a low ripening level: 2.5 (Garcia and Yousfi, 2005). Pieces of husk and stones in the OMSR were removed before characterisation and utilization in the experiments. The main characteristics and composition of the OMSR used in the experiments are shown in Table 1.

Equipment

A two-stage anaerobic digestion process was carried out in two different reactors at mesophilic temperature (35 ± 2 ºC). The first reactor or H-A reactor was a 1.5 L digester; it was initially loaded with 0.9 L of active biomass, 0.3 L of a nutrient-trace solution and 0.3 L of distilled water. The M reactor was loaded with 1 L of sludge, 0.4 L of a nutrient-trace element solution and 0.4 L of distilled water, keeping the effective reactor volume at 1.8 L. The nutrient-trace element solution has been previously described (Borja *et al.*, 2001). Trace metals are known to stimulate the activity of anaerobic microorganisms involved in the degradation of organic matter (Krongthamchat et al., 2006). To prevent the loss of microorganisms with the reactor effluents, the mixture contained in the M reactor was supplemented with saponite (0.8 g mL^{-1}), a low density magnesium silicate support media ($(Mg,Fe)_3(Si,Al)_4O_{10}(OH)_2.4H_2O$). An inoculum/support (saponite) ratio of 1:1 was kept. The M reactor was also equipped with a 0.5 L settler located on its upper part (Rincón *et al.*, 2009). Biogas was collected by a water displacement system fitted to the reactor (8 L Boyle-Mariotte reservoir). The CO_2 produced in the process was scrubbed by bubbling the gas mixture through a 3M NaOH solution. The volume of methane produced during the process was estimated by the volume of water displaced by this gas. Both reactors were continuously stirred at 260 rpm keeping an adequate inoculum-substrate mass transfer.

Inoculum

Both reactors (H-A and M) were inoculated with the same inoculum, which was an anaerobic sludge obtained from an industrial upflow anaerobic sludge blanket (UASB) reactor placed at a local brewery. Some of the characteristics of the inoculum used were: pH 8.1, TSS: 34.9, MSS: 8.9, VSS: 26.0, TS: 37.4, MS: 11.0 and VS: 26.4 g L^{-1} (TS: total solids, MS: total mineral solids, VS: total volatile solids, TSS: total suspended solids, MSS: mineral suspended solids, VSS: volatile suspended solids). All values were averages of triplicate samples with standard deviations lower than 5 %.

Experimental procedure

Before starting the experiments, the inoculum into the H-A and M reactors became acclimatized to the respective substrates OMSR and acidified OMSR. For the H-A reactor this adaptation period had a

double objective: an inoculum-substrate adaptation stage and an enrichment of the hydrolytic-acidogenic populations in the inoculum. The high susceptibility of the Archaea to high acidic concentrations prevented the coexistence of methanogens in the hydrolytic-acidogenic reactor. Therefore, the high duplication times of the methanogenic microorganisms and the short hydraulic retention times studied caused an enrichment of hydrolytic-acidogenic microorganisms in the H-A reactor.

The H-A reactor was adapted to the OMSR during 45 days, in this period the organic loading rate (OLR) was increased from 0.5 to 3 g COD L^{-1} d^{-1}. After that eight OLRs were studied. The study of these growing OLRs (OLRs of 3.2, 5.6, 7.4, 9.6, 11.0, 12.9, 14.0, and 15.1 g COD L^{-1} d^{-1}) in the H-A reactor enabled to determine the OLR of 12.9 g COD L^{-1} d^{-1} (equivalent to an hydraulic retention time (HRT) of 12.4 days) as the optimum OLR to lead the highest total volatile fatty acid (TVFA) concentration (Rincón *et al.,* 2008b). The effluents obtained at this OLR were used to feed the methanogenic reactor.

For the M reactor an adaptation of the inoculum to the acidified substrate coming from the H-A reactor was carried out with three different dilutions of acidified OMSR (25 %, 50 % and 75 %) for 45 days. The first dilution was used to keep the OLR between 0.5 and 1.5 g COD $L^{-1}d^{-1}$, the second to increase the OLR between 1.5 g and 2.2 g COD $L^{-1}d^{-1}$ and, finally, the third to increase the OLR to 3 g COD $L^{-1}d^{-1}$. After the inoculum became acclimatized to the acidified OMSR, OLRs of 0.8, 2.0, 3.5, 5.0, 6.5, 8.6, 10.5, 12.8, 14.0, 15.5, 17.0, 18.5 20.0 and 22.0 g COD $L^{-1}d^{-1}$ were assessed in the M reactor (HRTs between 142.9 and 4.6 days). Each OLR was kept during 2-3 hydraulic retention times, once the steady-state conditions were achieved for each run, and when the deviations between the observed values for consecutive measurements of some specific parameters (e.g. methane production, effluent CODs, etc.) were less than 5%, the samples were collected for analysis. Five different samples were taken over five consecutive days to ensure the representativeness of the data obtained for chemical analysis. 2 mL of these samples were collected to analyze the microbial community and were preserved at -80ºC until they were processed for DNA extraction.

Chemical analyses

The chemical analyses were performed according to the recommendations of the Standard Methods of APHA (APHA, 1989). COD and S-COD were determined according to the method number 5220 C. TS, MS, VS, TSS, MSS and VSS were analysed according to the method numbers 2540B and 2540E. TALK was determined using the 2320B method. The pH was determined with a Crison, model basic 20 pH-meter. Phosphorous was measured by spectrophotometry (880 nm) using the normalized methods 4500-P, B and E. Oils and fats were analysed by Soxhlet extraction with n-hexane using the official method of the EEC N^{o}2568/91 (European Community Official Diary, L248/1 of 05.09.1991). Poly-phenolic compounds were measured by spectrophotometry at 725 nm using the Folin-Ciocalteau method (Rincón *et al.,* 2007) after extraction following the procedure by Romero *et al.* (2002). Finally, TVFA were measured by distillation using the method number 5560C modified and titrating with sodium hydroxide.

Molecular characterization of microbial communities

The diversity of the microorganisms involved in the OMSR anaerobic treatment was assessed through culture-independent molecular techniques. DNA extraction was performed by using the Nucleospin Food DNA extraction kit (Macherey-Nagel, Düren, Germany) from steady-state samples. The microbial communities, both bacterial and archaeal, were analyzed through molecular fingerprinting and cloning of the 16S ribosomal RNA (16S rRNA) genes.

Each sample was represented by its microbial community fingerprint obtained by Denaturing Gradient Gel Electrophoresis (DGGE). DGGE was performed according to Muyzer et al. (1993) with the modifications incorporated by Portillo and González (2008) to obtain relative quantitative fingerprints from the analyzed samples. Polymerase Chain Reaction (PCR) amplification aimed for DGGE analysis was carried out with the primer pairs 341F-GC (5'-CCT ACG GGA GGC AGC AG containing a GC-rich tail at its 5' end) and 518R (5'-ATT ACC GCG GCT GCT GG) for bacterial sequences, and 344F-GC (5'-ACG GGG CGC AGC AGG CGC GA containing a GC-rich tail at the 5' end) and 518R for archaeal sequences (González and Sainz-Jimenez, 2004). The size of PCR products for DGGE analyses was approximately 160-190 bp. Amplification thermal conditions were: 95ºC for 2

min followed by 30 cycles of 95ºC for 15 s, 55ºC for 15 s, and 72ºC for 30 s; and a final step of 72ºC for 10 min. The GC-rich tail incorporated to the 5' end of the 341F-GC and 344F-GC primers was 5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G (Muyzer *et al.,* 1993).

For PCR amplification and cloning, the primer pairs 27F (5'-AGA GTT TGA TYM TGG CTC AG) and 1522R (5'-AAG GAG GTG ATC CAG CCG CA), for bacterial sequences, and 20bF (5'-YTC CSG TTG ATC CYG CSR GA) and 1492bR (5'-GGY TAC CTT GTK WCG ACT T), for archaeal sequences, were used (Orphan *et al.,* 2001). PCR thermal conditions were: 95ºC for 2 min followed by 30 cycles of 95ºC for 15 s, 55ºC for 15 s, and 72ºC for 2 min; and a final step of 72ºC for 10 min. The amplification products (approximately 1470-1500 bp) were used to construct 16S rRNA gene libraries aiming to identify the major components of the microbial communities. The 16S rRNA gene library construction was carried out with the TA-TOPO cloning kit (Invitrogen, Carlsbag, California) following the manufacturer recommendations. The screening procedure of library clones was carried out by DGGE and selected clones were sequenced. The major components of the microbial communities under analysis were selected by comparison of community fingerprints with the migration of the band corresponding to each clone (González *et al.,* 2003).

Homology searches based on the Blast algorithm (Altschul *et al.,* 1990) were performed online (http://www.ncbi.nlm.nih.gov/blast) to identify the closest relatives to the sequences retrieved during this study. Sequences were inspected for the presence of chimeras using the method described by González et al. (2005).

RESULTS AND DISCUSSION

After the acclimation period, OLRs of between 3.2 and 15.1 g COD L^{-1} d^{-1} were studied in the H-A reactor. The maximum TVFA concentration, 14.5 g CH_3COOH L^{-1} was searched for an OLR of 12.9 g COD L^{-1} d^{-1}. The microbial communities analyzed in the H-A step were the corresponding to OLRs of 11.0, 12.9 and 14.0 g COD $L^{-1}d^{-1}$, OLR values for which the highest TVFA concentrations were achieved. Fig. 1 shows the variation of the TVFA concentration with OLR in the H-A reactor and the M reactor. The variation of the TVFA with the OLR in a one-stage anaerobic digestion process,

previously studied by Rincón et al. (2007) is also shown. The TVFA concentration for all the OLRs studied in the M stage of the two-stage process was very low, under 1 g L^{-1} (as acetic acid), as shown in Fig. 1. Only for OLR higher than 20 g COD L^{-1} d^{-1}, the concentration increased until 3 g L^{-1} (as acetic acid). For the one-stage process, in a unique reactor (2007), TVFA concentrations over 1 g L^{-1} (as acetic acid) were achieved at lower OLR than in the two-stage process. For an OLR of 11 g COD L^{-1} d^{-1} the TVFA concentration was 1.4 g L^{-1}, causing a decrease in methane production. It seems that the acidification in a previous H-A reactor helps the consumption of the volatile fatty acids in the M reactor allowing to achieve higher OLR of work than in the digestion in one unique step.

For the M step, the microbial communities at OLRs of 2.0, 3.5, 6.5, 8.6, 10.5, 12.8, 15.5, 17.0, 18.5, 20.0 and 22.0 g COD $L^{-1}d^{-1}$were studied. The highest methane production rate, 3.24 L CH_4 $L^{-1}_{reactor}$ d^{-1}, was achieved in the M reactor for an OLR of 20.0 g COD $L^{-1}d^{-1}$ and 5 days of HRT. The methane yield obtained was 0.268±0.003 L CH_4 STP g^{-1} COD removed (Rincón *et al.,* 2009). This high and stable OLR achieved in the M reactor could be explained because the previous H-A stage behaved as an initial treatment of the OMSR, where the most complex compounds were hydrolyzed and solubilised. Among them a reduction of the poly-phenol compounds could be very beneficial before a second step as reported by other authors (Borja *et al.,* 1997).

The biotoxicity and inhibitory activity of phenols on the kinetics of acetoclastic methanogenesis is well known (Obied *et al.,* 2007). In the H-A reactor, working at an OLR of 12.9 g COD L^{-1} d^{-1}, a reduction of 40.7 % in the poly-phenolic concentration was achieved, when the highest TVFA production was achieved. Fig. 2 shows the poly-phenolic concentration as a function of the OLR in the H-A step and in the M step. When the concentration of poly-phenolic compounds reached values higher than 5 g L^{-1} (OLR>20 g COD L^{-1} d^{-1}) in the M reactor, the process started to fail and a reduction in the methane production yield was observed. For the study in one-stage (Rincón *et al.,* 2007), poly-phenolic concentrations higher than 4.4 g L^{-1} did fail the system but this concentration was obtained for an OLR of only 9.2 g COD L^{-1} d^{-1}. It is possible to conclude that the two-stage anaerobic treatment allows higher OLRs with improved stability than one-stage process. Similar results were reported by Hernandez and Edyvean (2004), a reduction of phenol concentration in a first acidogenic reactor, decreasing the toxic effect of phenols. In both cases, one stage and two-stage anaerobic

digestion processes with concentrations over 4.4-5.0 g L^{-1} of poly-phenolic compounds, resulted in the inhibition of the methanogenic Archaea and the methane produced in the M reactor was considerably reduced.

During this study, a total of 253 clones (200 bacterial and 53 archaeal) from 16S rRNA gene libraries were analyzed for the identification of the major components of the microbial communities developed in the reactors at different OLRs (Table 2). Fig. 3 shows the bacterial community fingerprints obtained by DGGE in the H-A reactor. This figure indicates the presence of highly dynamic microbial communities and intense microbial interactions dependent on the organic load existing in this reactor. Table 2 shows the correspondence between the identification letters (ID) used in Fig. 3, the classification of these microorganisms, their accession numbers, their closest homologues accession numbers and the percentages of similarity between the retrieved sequences and their closest relatives. The fractions represented by the major phylotypes detected during this analysis were obtained from quantitative DGGE analysis and they are shown in Tables 3 and 4 for Bacteria and Archaea, respectively. These results show a dynamic composition of the microbial communities at different OLR although the major components remained in the communities. In the present study the dominant microorganisms in the H-A reactor, at OLRs of 11.0, 12.9 and 14.0 g COD L^{-1} d^{-1} where the best acidification conditions were achieved, were the Firmicutes (from 31.1% to 61.1%, average 42.1%) mainly represented by Clostridiales, and the Chloroflexi (from 29.4% to 53.3%, average 47.35%) (Table 3). These two groups have been reported as being able to degradate a large number of organic compounds under a variety of conditions (Nelson *et al.,* 2011). Chloroflexi have been found in a wide range of anaerobic environments, including anaerobic bioreactors operating at both mesophilic and thermophilic temperatures (Chouari *et al.,* 2005). Cirne et al. (2007) found Firmicutes as the major group in the leachate fraction of two-stage anaerobic digestion reactors of energy crops. Firmicutes has been also reported as an important phylum into two-stage anaerobic digestion of food waste-recycling wastewater (Shin *et al.,* 2010). This bacterial phylum carries out a major role in OMSR degradation and it is able to outcompete with other microorganisms during the process. The degradation of OMSR might experience further improvements if additional studies are performed on these two bacterial groups which represent preferred targets for future optimizations of the studied

two-stage processes by selected enrichments of members of these two groups. Other microorganisms such as Gamma-Proteobacteria (*Pseudomonas* species as the major representative; from 3.9% to 9.7%; average 5.7%), Actinobacteria (from 1.0% to 10.2%; average 4.6%) and Bacteroidetes (from 1.1% to 3.1%; average 1.7%) were detected although represented lower fractions of the studied microbial communities (Table 3). Fig. 3 shows that for an OLR of 11 g COD L^{-1} d^{-1} the main microorganisms found were *Clostridium* (Firmicutes) (H and V), Chloroflexi (I and O) and *Paenibacillus* (N) (Table 2). But for the OLR showing the maximum generation of volatile fatty acids (12.9 g COD L^{-1} d^{-1}) Chloroflexi (K and O) and *Clostridium* (V) were the main bacteria and other members of the community drastically reduced their significance (i.e., H and N). These results suggest a progressive higher significance of Chloroflexi at increasing organic loads which represents an important finding from this study. The dominance of distinct phylotypes at different OLR values suggests that the organic matter added to the reactor per volume unit and time is an essential factor shaping microbial communities under development in OMSR treating reactors. The dominance of different microorganisms under distinct OLR suggests that the organic load and composition, as well as the initial inoculum composition (Moreno-Andrade and Buitrón, 2004; Nyholm *et al.*, 1984) could be decisive factors for optimum treatment results during the anaerobic digestion of OMSR. The TVFA concentrations achieved in the first stage (10.3, 14.5 and 13.0 g L^{-1} (as acetic acid)) led to a practical disappearance of methanogenic Archaea which were undetectable in the H-A reactor for the OLRs and HRTs studied. Other authors have not found a disappearance of the methanogenic microorganisms, although significant shifts after 13 days of operation have been reported (Liu *et al.*, 2002). These changes in the microbial community were accompanied with the increase in TVFA production and a decrease in methane formation. Variations in the morphology of autoflorescent methanogens depending on volatile fatty acid concentrations in the acid reactor has been described reporting that methanogens remained between 0.01-3 % of the total population (Ince and Ince, 2000).

The methanogenic communities detected in the M process were mainly represented by members of the archaeal genus *Methanosaeta* as shown in Fig. 4 and Table 2 (A', B', C' and D'). These results are in agreement to those obtained in single-stage anaerobic processes for OMSR degradation (Rincón *et al.*, 2008a) and in two-stage anaerobic processes treating residues of cassava meal industry (Paixao *et al.*,

2000). Methanosaeta has been suggested as the most tolerant methanogen to the inhibitory/toxic substances present in wastewaters such as olive oil mill wastewaters (García-García *et al.,* 2000; Tabatabaei *et al.,* 2010). The archaeal community fingerprints obtained by DGGE showed relatively constant profiles at most OLRs (2.0-18.5 g COD L^{-1} d^{-1}) (Fig. 4) where the reactor operation parameters were stable and the methane production was increasing. At the OLR with maximum methane production (20.0 g COD L^{-1} d^{-1}) the methanogenic phylotype C' was barely detected (Table 4). At higher OLRs, when the methane production decreased, there was a decrease in the proportion of the methanogenic phylotype D' (Table 4). This suggests its active role in reaching a maximum methane production and likely an inhibition of the growth at the high OLR tested. The possibility of these phylotypes being washed out is low because a support media (saponite) was used for microorganism immobilization with the aim of avoiding these losses. This dominant obligate acetotrophic methanogenic genus, *Methanosaeta*, was also found in a two-phase leach-bed system operating exclusively with triticale silage as the sole substrate. The detected Archaea were closely related to *M. concilii* (with 0.2-0.5 % of divergence) (Klocke *et al.,* 2008). Similar results were found by another study using 44 bioreactors with single-strand conformation polymorphism analyses. These reactors treated effluents from different origins such as agriculture, food processing, petro-chemical industries, pulp and paper plants, breweries, slaughterhouses and municipal wastes, and a vast number of existing reactor configurations were represented (fixed film, fluidized bed, stirred-tank, etc). In these cases, the most frequent (84 % of digesters) archaeal sequences were close relatives of *Methanosaeta concilii* (Leclerc *et al.,* 2004). *Methanosaeta* dominate in stable habitats, where the acetate levels are low, as they are specialist with a high affinity for acetate (McMahon *et al.,* 2001; Ma *et al.,* 2006). McHugh et al. (2003) found a dominance of *Methanosaeta* spp. in six different sludges, treating a variety of low- and high-strength, simple and complex wastewaters at psychrophilic (10-14ºC), mesophilic (37ºC) and thermophilic (55ºC) temperatures irrespective of wastewater type or operating temperature, indicating the importance of this organism in the stable and efficient operation of an anaerobic bioreactor (McHugh *et al.,* 2003).

CONCLUSIONS

The microbial populations involved in the two-stage OMSR anaerobic digestion were represented by dynamic communities which adapt to the degradable substrate and to the reactors conditions. Two major bacterial groups dominated in the H-A stage: the Clostridiales (Firmicutes) and the Chloroflexi. The *Methanosaeta* genus among the Archaea was the main methanogen during the M stage. Understanding the behaviour of microorganisms during the OMSR anaerobic treatment is important in order to obtain optimum results during the processing of these wastes. The knowledge of how the system works is decisive to optimize specific treatments and organic loads of anaerobic digesters for industrial application development.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to the Spanish "Ministerio de Educación y Ciencia" (Project REN 2001-0472/TECNO) and "Junta de Andalucía" for providing financial support and to the contracts JAE-Doc from "Junta para la Ampliación de Estudios del Consejo Superior de Investigaciones Científicas (CSIC)" co-financed by the European Social Funds.

REFERENCES

Altschul, S. F.; Gish, W.; Miller, W.; Myers, E. W.; Lipman, D. J., (1990). Basic local alignment search tool. J. Mol. Biol., 215, 403-410.

Amani, T.; Nosrati, M.; Mousavi, S. M.; Kermanshahi, R. K. (2011). Study of syntrophic anaerobic digestion of volatile fatty acids using enriched cultures at mesophilic conditions. Int. J. Environ. Sci. Technol., 8 (1), 83-96.

Anderson, G. K.; Kasapgil, B.; Ince, O., (1994). Microbiological study of two stage anaerobic digestion start-up. Water Res., 28, 2383-2392.

APHA, (1989). APHA (American Public Health Association), AWWA (American Water Works Association, WPCF (Water Pollution Control Federation). Standards Methods for the Examination of Water and Wastewater, 17th Ed. APHA, Washington, DC, USA.

Borja, R.; Alba, J.; Banks, C. J., (1997). Impact of the main phenolic compounds of olive mill wastewater (OMW) on the kinetics of acetoclastic methanogenesis. Process Biochem., 32, 121-133.

Borja, R.; González, E.; Raposo, F.; Millán, F.; Martín, A., (2001). Performance evaluation of a mesophilic anaerobic fluidized bed reactor treating wastewater derived from the production of proteins from extracted sunflower flour. Bioresour. Technol., 76, 45-52.

Borja, R.; Rincón, B.; Raposo, F., (2006). Anaerobic biodegradation of two-phase olive mill solid wastes and liquid effluents: Kinetic studies and process performance. J. Chem. Technol. Biotechnol., 81, 1450-1462.

Cirne, D.; Lehtomäki, A.; Björnsson, L.; Blackall, L., (2007). Hydrolysis and microbial community analyses in two-stage anaerobic digestion of energy crops. J. Appl. Microbiol., 103, 516-527.

Chen, C. L.;Wu, J. H.; Liu, W. T., (2008). Identification of important microbial populations in the mesophilic and thermophilic phenol-degrading methanogenic consortia. Water Res., 42, 1963-1976.

Chouari, R.; Le Paslier, D.; Daegelen, P.; Ginestet, P.; Weissenbach, J.; Sghir, A., (2005). Novel predominant archaeal and bacterial groups revealed by molecular analysis of an anaerobic sludge digester. Environ. Microbiol., 7, 1104-1115.

Demirel, B.; Yenigun, O., (2002). Two-phase anaerobic digestion processes: A review. J. Chem. Technol. Biotechnol., 77, 743-755.

Fang, H. H. P.; Liu, Y.; Ke, S. Z.; Zhang, T., (2004). Anaerobic degradation of phenol in wastewater at ambient temperatures. Water Sci. Technol., 49, 95-102.

García-García, I.; Jiménez-Peña, P. R.; Bonilla-Venceslada, J. L.; Martín-Martín, A.; Martín-Santos, M. A.; Ramos-Gómez, E., (2000). Removal of phenol compounds from olive mill wastewater using Phanerochaete chrysosporium, Aspergillus niger, Aspergillus terreus and Geotrichum candidum. Process Biochem., 35, 751-758.

Garcia, J. M.; Yousfi, K., (2005). Non-destructive and objective methods for the evaluation of the maturation level of olive fruit. Eur. Food Res. Technol., 221, 538-541.

González, J. M.; Ortiz-Martinez, A.; González-Delvalle, M. A.; Laiz, L.; Saiz-Jimenez, C., (2003). An efficient strategy for screening large cloned libraries of amplified 16S rDNA sequences from complex environmental communities. J. Microbiol. Methods, 55, 459-463.

González, J. M.; Sainz-Jimenez, C., (2004). Microbial diversity in biodeteriorated monuments as studied by denaturing gradient gel electrophoresis. J. Sep. Sci., 27, 174-180.

González, J. M.; Zimmermann, J.; Saiz-Jimenez, C., (2005). Evaluating putative chimeric sequences from PCR-amplified products. Bioinformatics, 21, 333-337.

Hernandez, J. E.; Edyvean, R. G., (2004). Anaerobic treatment of phenol in a two-stage anaerobic reactor. Waste Management and the Environment II. Proceeding of the Second International Conference on Waste Management and the Environment, ISBN: 1-85312-738-8, 203-211.

Ince, B. K.; Ince, O., (2000). Changes to bacterial community make-up in a two-phase anaerobic digestion system. J. Chem. Technol. Biotechnol., 75, 500-508.

Klocke, M.; Nettmann, E.; Bergmann, I.; Mundt, K.; Souidi, K.; Mumme, J.; Linke, B., (2008). Characterzation of methanogenic Archaea within two-phase biogas reactor systems with plant biomass. Syst. Appl. Microbiol., 31, 190-205.

Krongthamchat, K.; Riffat, R.; Dararat, S. (2006). Effect of trace metals on halophilic and mixed cultures in anaerobic treatment. Int. J. Environ. Sci. Technol., 3 (2), 103-112.

Leclerc, M.; Delgènes, J. P.; Gordon, J. J., (2004). Diversity of the archaeal community in 44 anaerobic digesters as determined by single strand conformation polymorphism analysis and 16S r DNA sequencing. Environ. Microbiol., 6, 809-819.

Liu, W.; Chan, O.; Fang, H., (2002). Microbial community dynamics during start-up of acidogenic anaerobic reactors. Water Res., 36, 3203-3210.

Ma, K.; Liu, X. L.; Dong, X. Z., (2006). Methanosaeta harundinacea sp. Nov., a novel acetate-scavening methanogen isolated from a UASB reactor. Int. J. Syst. Evol. Microbiol., 56, 127-131.

Mcmahon, K. D.; Stroot, P. G.; Mackie, R. I.; Raskin, L., (2001). Anaerobic codigestion of municipal solid waste and biosolids under various mixing conditions-II: Microbial population dynamics. Water Res., 35, 1817-1827.

Mchugh, S.; Carton, M.; Mahony, T.; O'flaherty, V., (2003). Methanogenic population structure in a variety of anaerobic bioreactors. FEMS Microbiol. Lett., 219, 297-304.

Moreno-Andrade, I.; Buitrón, G., (2004). Influence of the origin of the inoculum on the anaerobic biodegradability test. Water Sci. Technol., 49, 53-59.

Muyzer, G.; De Waal, E. C.; Uitterlinden, A. G., (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol., 59, 695-700.

Nelson, M. C.; Morrison, M.;Yu, Z., (2011). A meta-analysis of the microbial diversity observed in anaerobic digesters. Bioresour. Technol., 102, 3730-3739.

Nyholm, N.; Lindgaard-Jorgensen, P.; Hansen, N., (1984). Biodegradation of 4-nitrophenol in standardized aquatic degradation test. Ecotoxicol. Environ. Saf., 8, 451-470.

Obied, H. K.; Bedgood, J. D. R.; Prenzler, P. D.; Robards, K., (2007). Bioscreening of Australian olive mill waste extracts: Biophenol content, antioxidant, antimicrobial and molluscicidal activities. Food Chem. Toxicol., 45, 1238-1248.

Orphan, V. J.; Hinrichs, K. U.; Ussler Iii, W.; Paull, C. K.; Taylor, L. T.; Sylva, S. P.; Hayes, J. M.; Delong, E. F., (2001). Comparative Analysis of Methane-Oxidizing Archaea and Sulfate-Reducing Bacteria in Anoxic Marine Sediments. Appl. Environ. Microbiol., 67, 1922-1934.

Paixao, M. A.; Tavares, C. R. G.; Bergamasco, R.; Bonifacio, A. L. E.; Costa, R. T., (2000). Anaerobic digestion from residue of industrial cassava industrialization with acidogenic and methanogenic physical separation phases. Applied Biochemistry and Biotechnology - Part A Enzyme Engineering and Biotechnology, 84-86, 809-819.

Portillo, M. C.; González, J. M., (2008). Statistical differences between relative quantitative molecular fingerprints from microbial communities. Antonie Van Leeuwenhoek, 94, 157-163.

Rincón, B.; Borja, R.; González, J. M.; Portillo, M. C.; Sáiz-Jiménez, C., (2008a). Influence of organic loading rate and hydraulic retention time on the performance, stability and microbial communities of one-stage anaerobic digestion of two-phase olive mill solid residue. Biochem. Eng. J., 40, 253-261.

Rincón, B.; Sánchez, E.; Raposo, F.; Borja, R.; Travieso, L.; Martín, M. A.; Martín, A., (2008b). Effect of the organic loading rate on the performance of anaerobic acidogenic fermentation of two-phase olive mill solid residue. Waste Manag., 28, 870-877.

Rincón, B.; Borja, R.; Martín, M. A.; Martín, A., (2009). Evaluation of the methanogenic step of a two-stage anaerobic digestion process of acidified olive mill solid residue from a previous hydrolytic-acidogenic step. Waste Manag., 29, 2566-2573.

Rincón, B.; Travieso, L.; Sánchez, E.; De Los Ángeles Martín, M.; Martín, A.; Raposo, F.; Borja, R., (2007). The effect of organic loading rate on the anaerobic digestion of two-phase olive mill solid residue derived from fruits with low ripening index. J. Chem. Technol. Biotechnol., 82, 259-266.

Romero, C.; Brenes, M.; García, P.; Garrido, A., (2002). Hydroxytyrosol 4-B-D-glucoside, an important phenolic compound in olive fruits and derived products. J. Agric. Food Chem., 50, 3835-3839.

Shin, S. G.; Han, G.; Lim, J.; Lee, C.; Hwang, S., (2010). A comprehensive microbial insight into two-stage anaerobic digestion of food waste-recycling wastewater. Water Res., 44, 4838-4849.

Tabatabaei, M.; Rahim, R. A.; Abdullah, N.; Wright, A.-D. G.; Shirai, Y.; Sakai, K.; Sulaiman, A.; Hassan, M. A., (2010). Importance of the methanogenic archaea populations in anaerobic wastewater treatments. Process Biochem., 45, 1214-1225.

Veeken, A. H. M.; Hamelers, B. V. M., (2000). Effect of substrate-seed mixing and leachate recirculation on solid state digestion of biowaste. Water Sci. Technol., 41, 255-262.

Wang, Y. T.; Gabbard, H. D.; Pai, P. C., (1991). Inhibition of acetate methanogenesis by phenols. J. Environ. Eng., 117, 487-500.

Weiland, P., (2010). Biogas production: Current state and perspectives. Appl. Microbiol. Biotechnol., 85, 849-860.

Zhang, T.; Ke, S. Z.; Liu, Y.; Fang, H. P., (2005). Microbial characteristics of a methanogenic phenol-degradating sludge. Water Sci. Technol., 52, 73-78.

Figure Caption

Figure 1. Variation of the total volatile fatty acids concentrations in the hydrolityc-acidogenic step and in the methanogenic step of the two-stage anaerobic digestion process with the OLR. The variation of the total volatile fatty acids concentrations with the OLR in the one-stage OMSR anaerobic digestion obtained by Rincón et al. (2007) is also shown.

Figure 2. Variation of the poly-phenolic compounds in the hydrolityc-acidogenic step and in the methanogenic step of the two-stage anaerobic digestion process with the OLR.

Figure 3. DGGE fingerprints showing the major components of the bacterial communities developed in the H-A reactor at OLRs of 11.0, 12.9 and 14.0 g COD $L^{-1}d^{-1}$. The identified bacteria indicated on the right side with letters correspond to the listing and Accession no. provided in Table 2.

Figure 4. DGGE fingerprints showing the major components of the Archaea developed in the M reactor at OLR of 2.0, 3.5, 6.5, 8.6, 10.5, 12.8, 15.5, 17.0, 18.5, 20.0 and 22.0 g COD L^{-1} d^{-1} (listing and Accession numbers provided in Table 2).

Tables

Table 1. Characteristics of the two-phase OMSR used in the experiments.

Parameters	OMSR
pH	5.3
TALK (as $CaCO_3$)	1.1
TVFA (as acetic acid)	1.4
COD	162.0
S-COD	57.5
TS	143.0
MS	17.0
VS	126.0
TSS	106.0
MSS	11.0
VSS	95.0
Phosphorous	0.0035
Oils and fats	2.2 %
Moisture	86.7 %
Total phenols (as caffeic acid)	15.0

COD: total chemical oxygen demand, S-COD: soluble chemical oxygen demand, TVFA: total volatile fatty acids, TALK: total alkalinity, TS: total solids, MS: total mineral solids, VS: total volatile solids, SS: total suspended solids, MSS: mineral suspended solids, VSS: volatile suspended solids. All units are expressed in g L^{-1} except the moisture, oils, fats and pH. Values are averages of six determinations; there was virtually no variation (less than 5 %) between analyses.

Table 2. Bacteria and Archaea identified during this study and the number of clones obtained during the analysis of 16S rDNA gene libraries. ID letters correspond to the nomenclature of Fig. 2 and Fig. 3 for Bacteria and Archaea, respectively.

		Bacteria			
ID	**Identified bacterial group/genus**	**GenBank Accession No.**	**Closest homologue Acc. No.**	**% Similarity**	**Number of Clones**
A	Chloroflexi	**AY935682**	**AJ249111**	**98**	**5**
B	Chloroflexi	**AY935680**	**AJ249111**	**99**	**1**
C	Chloroflexi	**AY935673**	**AF323767**	**98**	**2**
D	*Clostridium* (Firmicutes)	**AY935677**	**AY386136**	**99**	**2**
E	*Bacillus* (Firmicutes)	**AY935679**	**AJ277983**	**99**	**1**
F	Chloroflexi	**AY935664**	**AJ249111**	**99**	**25**
G	Chloroflexi	**AY935665**	**AJ249111**	**99**	**9**
H	*Clostridium* (Firmicutes)	**AY935683**	**AJ009459**	**93**	**6**
I	Chloroflexi	**AY935659**	**AJ249111**	**98**	**35**
J	*Clostridium* (Firmicutes)	**AY935661**	**AY386236**	**98**	**29**
K	Chloroflexi	**AY935666**	**AJ249111**	**97**	**7**
L	Chloroflexi	**AY935667**	**AJ009488**	**98**	**8**
M	*Pseudomonas* (Gammaproteobacteria)	**AY935658**	**AJ278174**	**91**	**1**
N	*Paenibacillus* (Firmicutes)	**AY935675**	**U81668**	**99**	**1**
O	Chloroflexi	**AY935653**	**AF424402**	**96**	**11**
P	*Pseudomonas* (Gammaproteobacteria)	**AY935662**	**AF289505**	**99**	**18**
Q	Chloroflexi	**AY935654**	**AJ249193**	**99**	**1**
R	*Clostridium* (Firmicutes)	**AY935681**	**AB088977**	**92**	**2**
S	Chloroflexi	**AY935668**	**AJ306792**	**97**	**1**
T	Gammaproteobacteria	**AY935671**	**AF332298**	**96**	**3**
U	*Clostridium* (Firmicutes)	**AY935678**	**AY360624**	**99**	**1**
V	*Clostridium* (Firmicutes)	**AY935674**	**AJ506119**	**98**	**28**
W	*Rubrobacter* (Actinobacteria)	**AY935656**	**AB116447**	**97**	**2**
X	*Bacteroides* (Bacteroidetes)	**AY935672**	**AB186816**	**97**	**1**
				Total	**200**
		Archaea			
ID	**Identified archaeal group/genus**	**GenBank Accession No.**	**Closest homologue Acc. No.**	**% Similarity**	**No. Clones**
A’	*Methanosaeta*	**AY935685**	**AY692054**	**99**	**18**
B’	*Methanosaeta*	**AY935687**	**AY570662**	**99**	**15**
C’	*Methanosaeta*	**AY935686**	**AY692054**	**99**	**7**
D’	*Methanosaeta*	**AY935688**	**AY835796**	**99**	**13**
				Total	**53**

Table 3. Fractions (%) of the bacterial OTUs identified in this study from quantitative community fingerprinting analysis. ID letters correspond to the nomenclature of Fig. 2 and Table 2.

	OLR (g COD L^{-1} d^{-1})			
ID	**11.0**	**12.9**	**14.0**	**Avg (sd)[1]**
A	6.02	12.21	3.21	7.14 (4.60)
B	1.98	2.34	16.67	6.99 (8.38)
C	3.13	2.61	0.87	2.20 (1.18)
D	1.44	0.25	16.00	5.90 (8.77)
E	9.86	0.65	0.14	3.55 (5.47)
F	1.75	5.92	3.90	3.85 (2.08)
G	1.86	3.51	0.07	1.81 (1.71)
H I	8.89	2.63	9.58	7.03 (3.83)
J	0.16	6.79	8.43	5.12 (4.38)
K	0.06	9.20	nd[2]	4.60 (6.50)
L	0.82	2.23	20.40	7.82 (10.93)
M	0.43	1.82	7.67	3.30 (3.84)
N	15.28	4.36	0.86	6.83 (7.50)
O	4.98	11.61	2.14	6.25 (4.85)
P	2.80	0.62	0.73	1.38 (1.22)
Q	3.63	0.67	2.44	2.25 (0.98)
R	0.85	nd[2]	1.89	0.91 (0.95)
S	0.74	1.67	0.40	0.93 (0.66)
T	0.68	2.15	0.32	1.05 (0.97)
U	nd[2]	6.44	1.09	2.51 (3.44)
V	29.06	11.25	1.04	13.78 (14.17)
W	2.51	10.22	1.05	4.60 (4.93)
X	3.06	0.85	1.10	1.67 (1.20)
Total	100	100	100	

[1] Average (Standard deviation).

[2] nd, not detected by DGGE fingerprinting

Table 4. Fractions (%) of the archaeal OTUs identified in this study from quantitative community fingerprinting analysis. ID letters correspond to the nomenclature of Fig. 3 and Table 2.

	OLR (g COD L^{-1} d^{-1})											
ID	**2.0**	**3.5**	**6.5**	**8.6**	**10.5**	**12.8**	**15.5**	**17.0**	**18.5**	**20.0**	**22.0**	**Avg (sd)[1]**
A’	47.63	40.54	34.55	54.71	23.73	28.61	33.81	37.92	46.29	51.30	58.23	41.57 (11.02)
B’	36.70	30.03	37.02	25.26	35.93	36.76	34.94	17.34	16.06	26.61	31.13	29.80 (7.68)
C’	8.80	3.07	9.89	1.38	26.02	23.40	15.39	21.68	10.74	1.02	1.87	11.21 (6.74)
D’	6.87	26.36	18.54	18.65	14.32	11.23	15.86	23.06	26.91	21.07	8.77	17.42 (6.74)
Total	100	100	100	100	100	100	100	100	100	100	100	

[1] Average (Standard deviation).

Figures

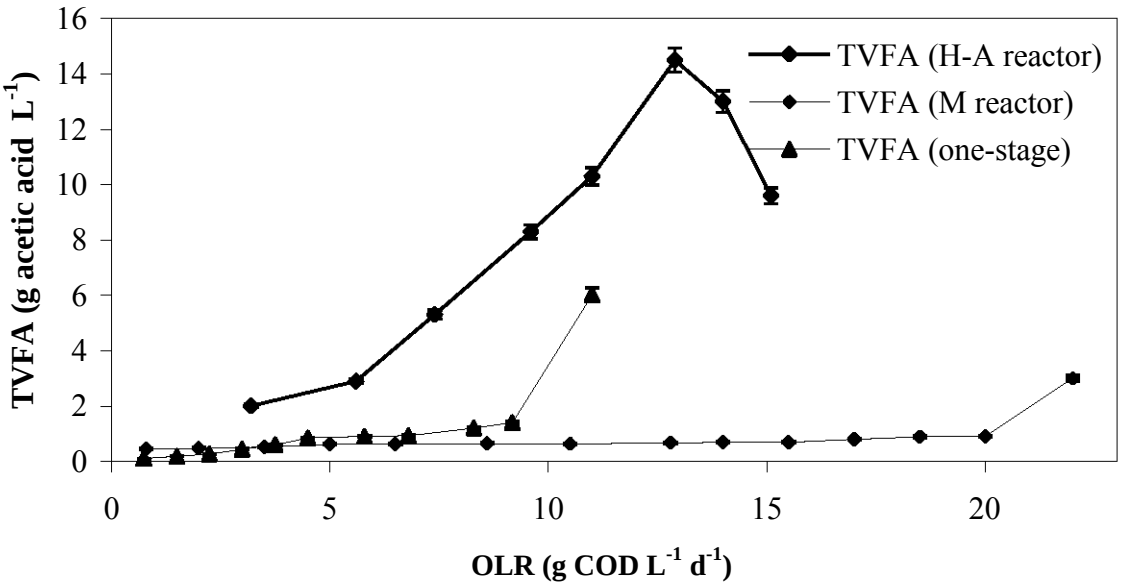

Fig. 1

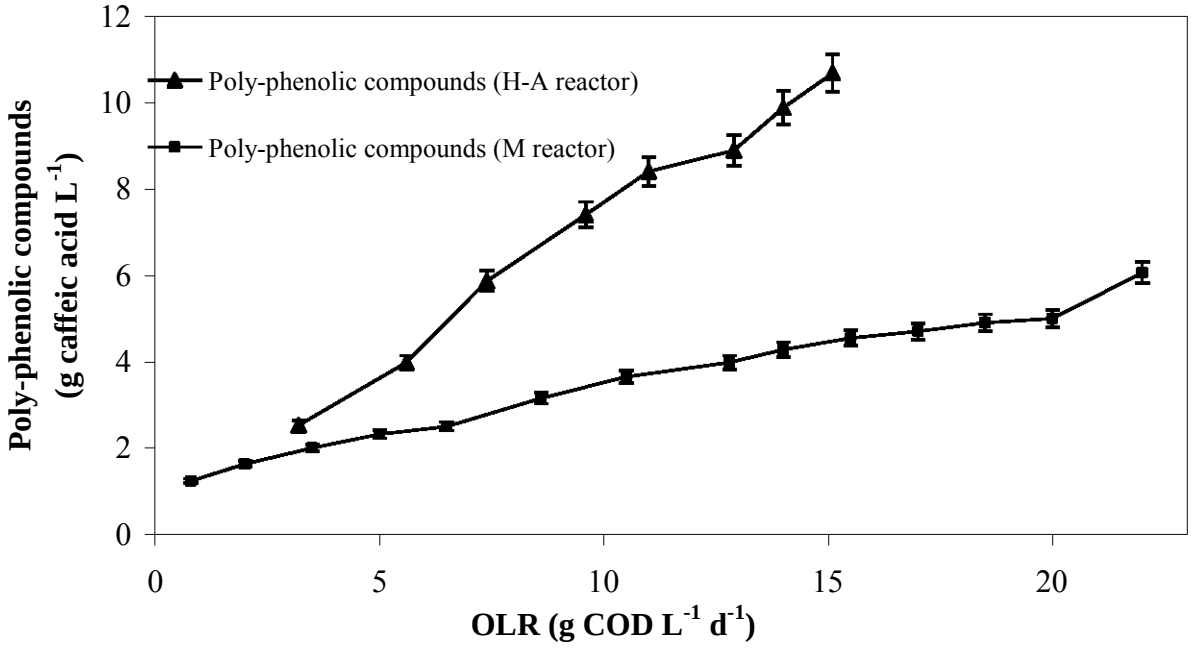
Poly-phenolic compounds (H-A reactor)
Poly-phenolic compounds (M reactor)
Poly-phenolic compounds (g caffeic acid L-1)
OLR (g COD L-1 d-1)
0
2
4
6
8
10
12
0
5
10
15
20

Fig. 2

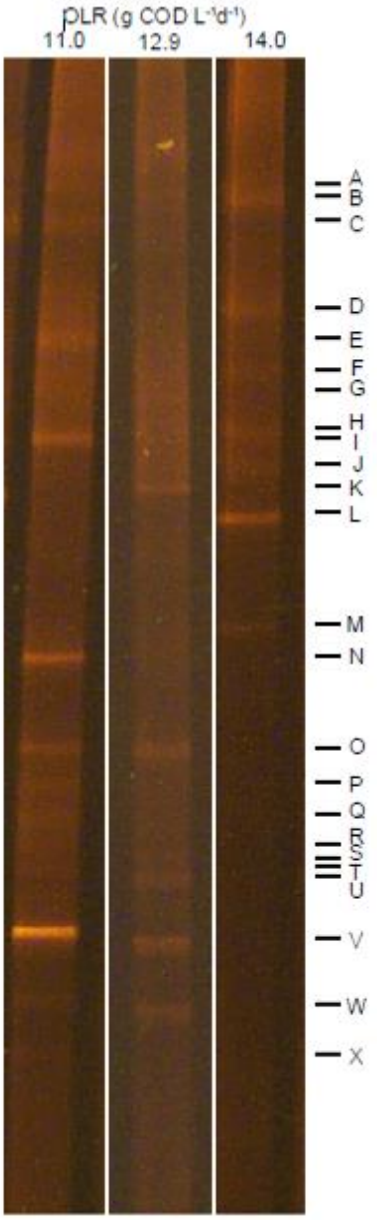

Fig.3

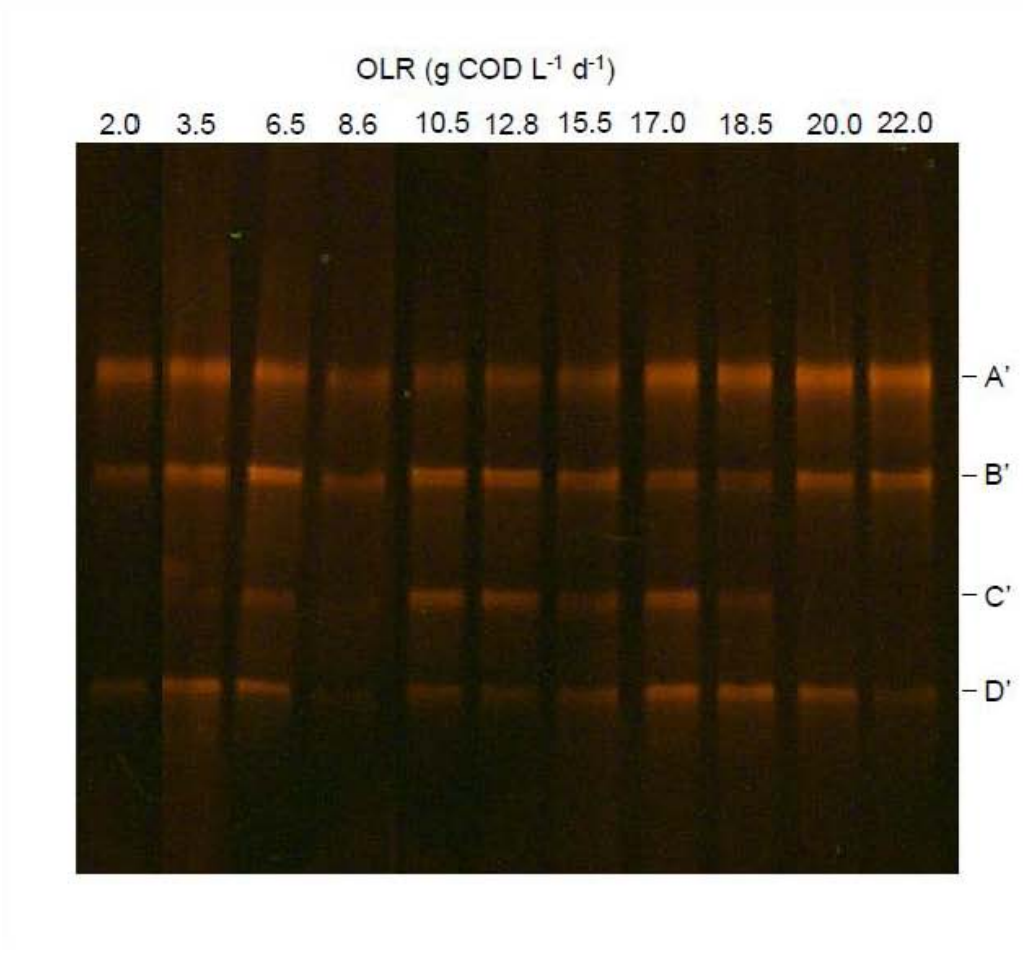

Fig. 4