

Molecular microbial and chemical investigation of the bioremediation of two-phase olive mill waste using laboratory-scale bioreactors

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Abstract Two-phase olive mill waste (TPOMW) is a semi-solid effluent that is rich in contaminating polyphenols and is produced in large amounts by the industry of olive oil production. Laboratory-scale bioreactors were used to investigate the biodegradation of TPOMW by its indigenous microbiota. The effect of nutrient addition (inorganic N and P) and aeration of the bioreactors was studied. Microbial changes were investigated by PCR-temperature time gradient electrophoresis (TTGE) and following the dynamics of polar lipid fatty acids (PLFA). The greatest decrease in the polyphenolic and organic matter contents of bioreactors was concomitant with an increase in the PLFA fungal/bacterial ratio. Amplicon sequences of nuclear ribosomal internal transcribed spacer region (ITS) and 16S rDNA allowed identification of fungal and bacterial types, respectively, by comparative DNA sequence analyses. Predominant fungi identified included members of the genera *Penicillium*, *Candida*, *Geotrichum*, *Pichia*, *Cladosporium*, and *Aschochyta*. A total of 14 bacterial genera

were detected, with a dominance of organisms that have previously been associated with plant material. Overall, this work highlights that indigenous microbiota within the bioreactors through stimulation of the fungal fraction, is able to degrade the polyphenolic content without the inoculation of specific microorganisms.

Keywords Bioremediation · Microbial diversity · Olive mill waste

Introduction

The olive oil industry generates large amounts of byproducts that are harmful to the environment. Olive mill wastes (OMWs) contain phytotoxic components capable of inhibiting microbial growth (Capasso et al. 1995; Ramos-Cormenzana et al. 1996) and the germination and vegetative growth of plants (Linares et al. 2003). Traditionally, the disposal of OMWs has become a great problem in Mediterranean countries because of their polluting effects on soil and water (Sierra et al. 2001; Piotrowska et al. 2006). The production of olive oil is increasing worldwide, with a growth rate that is expected to be between 3.5 and 4% per year, according to the International Olive Oil Council (Anonymous 2004). Moreover, this production is no longer restricted to the Mediterranean Basin, and new producers such as Australia, USA, and South American countries will have to face the environmental problems posed by OMWs.

At present, three systems are used worldwide for the industrial-scale extraction of oil from olives, viz. the traditional press-cake system, the three-phase decanter system and the modern two-phase centrifugation system. The present paper focuses on the two-phase centrifugation

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system, which was introduced in the 1990s as an ecological approach for olive oil production, since it drastically reduces water consumption during the process. The waste stream of this system consists of a semi-solid waste: the two-phase olive mill waste (TPOMW) or alpeorujo. The resulting waste comprises about 800 kg per 1,000 kg of the processed olives, and its production may exceed 4 million tons annually in Spain alone (Aragón and Palancar 2001; Albuquerque et al. 2004). The TPOMW consists of a thick sludge that contains water and pieces of pit plus the pulp of the olive fruit. This semi-solid effluent has a water content of about 65% (Arjona et al. 1999), a slightly acidic pH, and a very high content of organic matter, mainly composed of lignin, hemicellulose and cellulose; it also has a considerable proportion of fats, proteins, water-soluble carbohydrates, and a small but active fraction of hydro-soluble (poly)phenolic substances (Albuquerque et al. 2004).

The polyphenol content of olive residues consists of a complex mixture of compounds and is considered to be the most problematic fraction of OMWs, being associated with the well-known antimicrobial and phytotoxic effects of these residues (Ramos-Cormenzana et al. 1996). Bioremediation is a valuable tool for the detoxification of TPOMW by breaking down these phenolic compounds. Recent research on TPOMW bioremediation has included different approaches, such as anaerobic digestion (Borja et al. 2005; Rincón et al. 2006), natural biodegradation in evaporation ponds (Buyer et al. 1999), removal of phenols by saprophytic fungi (Sampedro et al. 2004), biodegradation by co-composting with agricultural wastes (Paredes et al. 2002), vermicomposting (Benitez et al. 2005), and the production of metal-binding microbial exopolysaccharides (Morillo et al. 2006). Despite the importance of bioremediation as an alternative to TPOMW detoxification and recycling, surprisingly little information is available on the indigenous microbiota of the residue and their potential for carrying out biodegradation of the waste (Jones et al. 2000; Giannoutsou et al. 2004; Ntougias et al. 2006). To our knowledge, the only detailed molecular characterization of the microbial communities of TPOMW is that of Rincón et al. (2006), where the molecular identification of the microbial species involved in a process of anaerobic treatment of diluted TPOMW was performed by PCR-DGGE. The Firmicutes (mainly represented by Clostridiales) were the most abundant phylotype, followed by the Chloroflexi and the Gammaproteobacteria (*Pseudomonas* species; Rincón et al. 2006).

Thus, the aim of the present investigation was twofold: first, to evaluate at laboratory scale the effect of nutrient amendment and aeration on the bioremediation of TPOMW, and second, to characterize the indigenous microbial communities present in TPOMW (i.e., bacteria

and fungi) and to elucidate how they change in response to nutrient amendment and aeration. The results of this study establish the foundations of a systematic study to identify optimal bioremediation conditions.

Materials and methods

Two-phase olive mill waste The TPOMW was obtained from the factory “Aceites Jimena S.A.” (Granada, Spain), collected in sterile plastic containers and stored at -20°C . The sample was dark brown in color and had a smooth dough-like consistency, with a high content of water (56%) and organic matter (41.14%), slightly acidic pH (5.53) and a high C/N ratio (40.77 mol/mol).

Experimental design and sampling Four experimental conditions were tested in triplicate using 63 in-vessel glass bioreactors (each 200 ml) at 25°C . For each bioreactor, TPOMW, water, and wheat straw at a ratio of 15:18:2.4, by weight basis was thoroughly mixed in a glass beaker (500 ml), and then the mixture introduced into the reactor. Initial moisture content (MC) of the mixture was measured, and double distilled water (DDW) was added to reach 65% MC (Antizar-Ladislao et al. 2006). Compost moisture content was measured at intervals to ensure that it was maintained at the required level and amended with DDW when needed. The logistic approach to pinpoint the effect of nutrient amendment and aeration on the bioremediation of TPOMW and microbial community changes was to investigate four different scenarios: non-aerated (OA), non-aerated + nutrients (OAN), aerated (AA), and aerated + nutrients (AAN). For the aerated treatments (AA and AAN), the reactor units stood vertically with air flowing continuously up through the mixture to avoid oxygen limitation. The air was vented outdoors to avoid accumulation of volatile compounds in the bioreactors. Airflow through the mixture was achieved by means of a stainless steel air-delivery tube inserted into the bottom of the bioreactors attached by silicone tubing to a 100% oil-free diaphragm pump (Model PXW-600-DIOV, VP1, 5 l min^{-1} , Fisher Scientific). The air inlet was bubbled through a DDW reservoir to avoid excessive water evaporation during aeration at 12 h intervals on and off. For the non-aerated bioreactors (OA and OAN), the set-up was exactly the same, except that the bioreactors were not aerated. For the nutrient-amended bioreactors (AAN and OAN), the mixture in each bioreactor was amended at the beginning of the experiment with 1 g of nitrogen and 50 mg of phosphorus, using NH_4NO_3 and KH_2PO_4 , respectively. Destructive sampling, in triplicate, for each bioreactor mixture was done after 0, 7, 14, 21, 35, 55, and 77 days. The contents of each bioreactor were thoroughly mixed in a 500-ml beaker,

and different subsamples were collected. Moisture content, ash content, and pH were analyzed on the same day of the sampling. Subsamples for polyphenol content determinations and microbial community analysis were kept frozen (-20°C) in sterile containers.

Determination of moisture content and total organic matter Total organic matter (TOM) was determined by ashing using a loss-on-ignition procedure. Triplicate 2 g samples were dried for 24 h at 110°C and reweighed to give the moisture content. The dried samples were transferred to a muffle furnace held at 550°C for 12 h. Ash content was calculated from the ratio of pre-ignition and post-ignition sample weights. The TOM losses were calculated as described previously (Albuquerque et al. 2006).

Polyphenol analysis Extraction of polyphenolic substances from the mixture was performed as follows. Approximately 1 g of sample was weighed and extracted with 20 ml of methanol/water (20:80, v/v) using an orbital shaker (150 rpm) for 2 h at 25°C . A modification of the Folin–Ciocalteu reagent assay was used to determine the total phenolic content (Maestro-Durán et al. 1991). All the assays were performed in triplicate. Total amount of phenolic compounds was calculated and expressed as caffeic acid-equivalent, CAE (mg g^{-1}).

Polar lipid fatty acid analysis of the microbial community Polar lipids were extracted from mixture samples (1 g) using a modified Bligh and Dyer method and their fatty acyl chains converted to fatty acid methyl esters (FAMES) by transmethylation using 2.5% (v/v) sulfuric acid in dry methanol (Kates 1985). The FAMES were analyzed by GC-MS using a Hewlett Packard 6890 series gas chromatograph and a 7673 series auto-sampler and a 5973 series mass selective detector, as previously described (Antizar-Ladislao et al. 2007). The sum of the following fatty acids was used to represent total bacteria: i15:0, a15:0, i16:0, i17:0, cy17:0, 18:1 ω 7c, and cy19:0 (Frostegård and Bååth 1996). Gram-positive bacteria were represented by i15:0, a15:0, and i17:0 (Buyer et al. 1999) and Gram-negative bacteria by cy17:0, 18:1 ω 7c, and cy19:0 (Klamer and Bååth 1998). Thermophilic bacteria (largely thermotolerant bacilli) were represented by i15:0 and i17:0 (Carpenter-Boggs et al. 1998). Fungi were represented by 18:2 ω 6,9 (Frostegård and Bååth 1996).

Molecular characterization of the microbial community
1. **DNA isolation and 16S rRNA gene (bacterial) and ITS2 (fungal) PCR amplification.** Total DNA was isolated from the samples employing the PowerSoil™ DNA isolation kit (MoBio Laboratories) following the manufacturer's instructions. Following electrophoresis on a 1% agarose gel

containing ethidium bromide, the integrity of bands was assessed visually and the concentration of nucleic acids measured spectrophotometrically. Primers U968-GC (5'-CGC CCG GGG CGC GGC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC) and L1401 (5'-GCG TGT GTA CAA GAC CC) were used to amplify the V6–V8 region of the bacterial 16S rRNA gene, giving a product of 450 bp. The ITS2 region of fungal genomic DNA was amplified with the primer pair ITS3-GC (5'-CGC CCG GGG CGC GGC CCG GGC GGG GCG GGG GCA CGG GGC ATC GAT GAA GAA CGC AGC) and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC), resulting in 370 bp product (Nicolcheva et al. 2005; White et al. 1990). PCR was performed using Hot Star Taq DNA polymerase (QIAGEN, Courtaboeuf, France). PCR mixtures (25 μl) contained the following: 1 \times PCR buffer, 1.5 mM MgCl_2 , 0.1 mM of each dNTP, 0.5 μM of primers, 2.5 U of Hot Star Taq polymerase, and approximately 1 ng of DNA. The DNA fragments were amplified using a GeneAmp PCR System 2700 (Applied Biosystem, Singapore). The following program was used with the primers U968-GC and L-1401: 95°C for 15 min; 30 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min 30 s, and finally 72°C for 15 min. Amplification of genomic DNA with the primers ITS3-GC and ITS4 was performed with the following program: 94°C for 15 min; 35 cycles of 94°C for 30 s, 47°C for 1 min, and 72°C for 1 min, and finally 72°C for 10 min. Negative (without DNA) controls were used in every series of reaction. Amplicons were analyzed by electrophoresis on a 1.5% agarose gel containing ethidium bromide to check the correct size and concentration.

2. **TTGE analysis of PCR amplicons.** The Dcode Universal Mutation Detection System (Bio-Rad, Paris, France) was used for sequence-specific separation of PCR products. Electrophoresis was performed through a 1-mm-thick, 16×16 cm polyacrylamide gel [8% (w/v) acrylamide–bisacrylamide, 7 M urea, 1.25 \times Tris–acetate–EDTA (TAE), 55 and 550 μl of Temed, and 10% ammonium persulfate] using 7 l of 1.25 \times TAE as electrophoresis buffer. Electrophoresis was performed at a fixed voltage of 65 V for 969 min with an initial temperature of 66°C and a ramp rate of 0.2°C per hour. For better resolution, the voltage was fixed at 20 V for 5 min at the beginning of electrophoresis. Each well was loaded with 100 to 200 ng of amplified DNA plus an equal volume of 2 \times gel loading dye (0.05% bromophenol blue, 0.05% xylene cyanol, and 70% glycerol). After completion of electrophoresis, the gel was stained in a SYBR Green I solution (Sigma-Aldrich, St. Quentin Fallavier, France), and destained in 1.25 \times TAE. Gels were visualized under UV illumination using a gel image system (Kodak Gel Logic 100). Predominant bands were excised and treated for further analysis.

3. *Comparison of TTGE patterns.* The digital images of the acrylamide gels were analyzed as previously described (Marie et al. 2006) using the Cross-Checker software (available from <http://www.dpw.wau.nl/pv/pub/CrossCheck/>).

4. *Sequence analysis.* To perform sequence-based phylogenetic identification, specific bands were cut from the polyacrylamide gel. Gel fragments were washed once in 200 µl of PCR water and kept in 100 µl of PCR water overnight at 4°C for diffusion. Both bacterial 16S rRNA and fungal ITS2 gene fragments were then amplified from the dialysate. The PCRs were performed in the same conditions as described above. The amplicons were purified using the GFX PCR DNA Purification Kit (Amersham Biosciences), and the size and concentration of the amplicons were evaluated on 1.5% agarose gels containing ethidium bromide. PCR products were sequenced with an Applied Biosystems 373A DNA sequencer by automated Sanger method. Similarity searches with sequences in the GenBank database were performed by using Blast algorithm.

5. *Nucleotide sequence accession numbers.* The ITS-2 region gene (fungal) and 16S region rRNA gene (bacterial) amplicon sequences identified in each treatment have been submitted to GenBank and the accession numbers are shown in Tables 1 and 2.

Statistical analysis The effect of different treatments on polyphenols biodegradation was investigated using a post hoc Tukey test. All the statistical tests were performed with Statistix Version 1.8.

Results

Chemical changes The changes in concentration of polyphenols are shown in Fig. 1a. Generally, there was greater biodegradation of polyphenols during the first few days of the experiment (depending on the treatment), and thereafter, their loss was slow. The greatest amount of biodegradation was in aerated bioreactors nutrient amended, in which there was 36% and 54% losses after 7 and 55 days, respectively. The biodegradation in aerated and not aerated bioreactors that were not amended with nutrients was not significant ($p > 0.1$). The addition of nutrients stimulated the biodegradation of polyphenols during the first 7 days of treatment under both aerated and non-aerated conditions ($p < 0.005$; Fig. 1a); however, after 55 days, this stimulation was only apparent in the aerated bioreactors, and after 77 days, the biodegradation curves reached a plateau (data not shown). Following 55 days of continuous treatment in non-aerated bioreactors, approximately 22% of polyphenols were lost, irrespective of whether they were amended with nutrients.

The bulk (about 95%) of the bioreactor material was organic matter, measured as total organic matter (TOM), and the losses over a 55-day incubation period are shown in Fig. 1b. Both aeration and nutrient addition influenced the rate of TOM losses. The addition of nutrient stimulated TOM loss in aerated bioreactors but decreased TOM loss in non-aerated bioreactors. In all bioreactors, the rate of loss of TOM was faster during the initial 14 days of incubation. Thereafter, the rate of loss was slow. Following 55 days of

Table 1 Bacteria identified by PCR-TTGE analysis in TPOMW bioreactors according to their similarity to 16S rRNA gene sequences in the GenBank nucleotide sequence database

Band no.	Treatment	Closest match (accession no.)	Percent similarity	Accession no.
<i>Gamma proteobacteria</i>				
OAN15	Non-aerated + nutrients	<i>Stenotrophomonas maltophilia</i> (AM261992)	94	EU450807
OAN18	Non-aerated + nutrients	<i>Erwinia persicina</i> (AJ937837)	97	EU540808
AA1	Aerated	<i>Luteibacter sp.</i> (DQ986460)	99	EU450809
AA3	Aerated	<i>Stenotrophomonas maltophilia</i> (AM262002)	99	EU4508010
<i>Actinobacteria</i>				
OA7	Non-aerated	<i>Rhodococcus fascians</i> (AY730713)	98	EU4508011
OAN8	Non-aerated + nutrients	<i>Clavibacter michiganensis</i> (L43096)	97	EU4508012
OAN10	Non-aerated + nutrients	<i>Frigoribacterium sp.</i> (AY439250)	97	EU4508013
OAN9	Non-aerated + nutrients	<i>Curtobacterium albidum</i> (AM042692)	95	EU4508014
<i>Firmicutes</i>				
AA6	Aerated	<i>Clostridium saccharolyticum</i> (AY604565)	98	EU4508015
AA7	Aerated	<i>Clostridium xylanolyticum</i> (X71855)	98	EU4508016
AA8	Aerated	<i>Clostridium sp.</i> (AB114227)	97	EU4508017
OA3	Non-aerated	<i>Clostridium tyrobutyricum</i> (L08062)	93	EU4508018
OA6	Non-aerated	Uncultured <i>Clostridium sp.</i> (DQ168846)	98	EU4508019
OA2	Non-aerated	<i>Lactobacillus vaccिनosterus</i> (AB218801)	99	EU4508020
OA4	Non-aerated	<i>Leuconostoc mesenteroides</i> (EF068254)	98	EU4508021
OA5	Non-aerated	<i>Leuconostoc mesenteroides</i> (EF068254)	99	EU4508022
D10	Initial mixture	<i>Bacillus aquimaris</i> (DQ923229)	97	EU4508023
OA1	Non-aerated	<i>Sporolactobacillus inulinus</i> (D16283)	98	EU4508024

Table 2 Fungi identified by PCR-TTGE analysis in TPOMW bioreactors according to their similarity to ITS2 region sequences in the GenBank nucleotide sequence database

Band no.	Treatment	Closest match (accession no.)	Percent similarity	Accession no.
AAN1	Aerated + nutrients	<i>Penicillium roquefortii</i> (DQ148947)	99	EU450825
AAN4	Aerated + nutrients	<i>Penicillium roquefortii</i> (AY373929)	99	EU450826
AAN6	Aerated + nutrients	<i>Penicillium roquefortii</i> (AY373929)	97	EU450827
AAN7	Aerated + nutrients	<i>Candida norvegica</i> (DQ249194)	92	EU450828
AAN10	Aerated + nutrients	<i>Geotrichum</i> sp. (AY787702)	100	EU450829
OAN12	Non-aerated + nutrients	<i>Pichia membranifaciens</i> (DQ104713)	99	EU450830
OAN15	Non-aerated + nutrients	<i>Cladosporium herbarum</i> (AY251078)	100	EU450831
OAN17	Non-aerated + nutrients	<i>Ascochyta</i> sp. (AY305377)	100	EU450832
OAN19	Non-aerated + nutrients	<i>Geotrichum</i> sp. (AY787702)	99	EU450833
OAN20	Non-aerated + nutrients	<i>Geotrichum</i> sp. (AY787702)	99	EU450834

incubation, the loss of TOM was in the range of 10–25% in all bioreactors, and greatest losses were observed in nutrient amended aerated bioreactors (Fig. 1b).

Microbial community structure

1. *Phospholipid fatty acid (PLFA) analysis.* The changes in relative amounts of fungal and bacterial biomass in the bioreactors during 55 days incubation are shown in Fig. 2. The addition of nutrients increased the fungal/bacterial PLFA index (F/B index) in both aerated and

non-aerated bioreactors, the greatest changes occurring during the initial 14 days of incubation, and generally being slow or static thereafter. The increase in F/B index was greater in the nutrient amended aerated bioreactors, in which the presence of fungal biomass was sufficient to be seen as white mycelium by simple visual observation: this was not seen in any other bioreactor conditions, even in the nutrient amended non-aerated bioreactors, which also displayed a steady rise in the F/B index throughout the 55 days incubation period (Fig. 2). In contrast, in both aerated and non-aerated bioreactors without nutrient amendment, there was a decrease in the F/B index. However, the kinetics of the decrease differed: in the aerated bioreactors, the decrease occurred during the initial 7 days of incubation, in comparison with the non-aerated bioreactors in which it occurred during the latter half of the incubation period (Fig. 2).

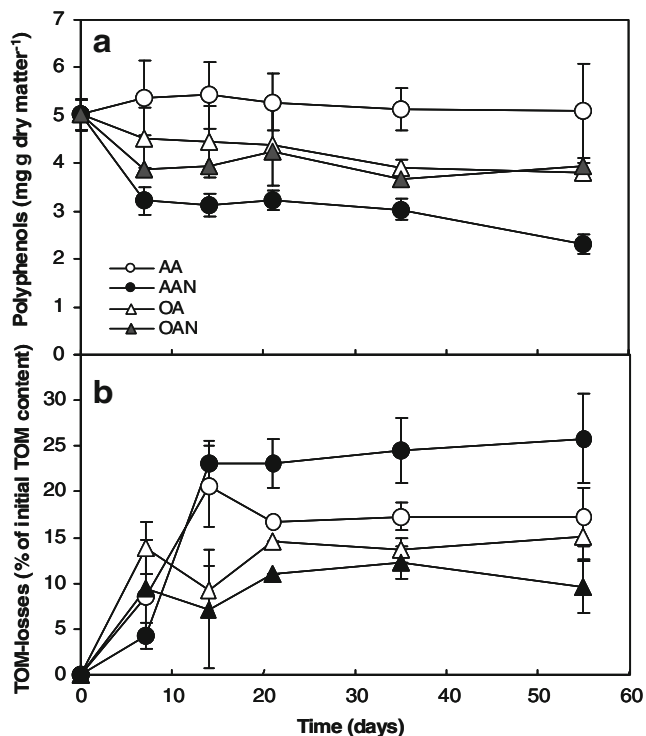


Fig. 1 Polyphenol biodegradation (a) and organic matter losses (b). The data points are mean values of samplings from triplicate reactors (bars correspond to \pm standard deviation) on the days shown. Symbols refer to the different treatments: aerated (AA), aerated + nutrients (AAN), non-aerated (OA) and non-aerated + nutrients (OAN)

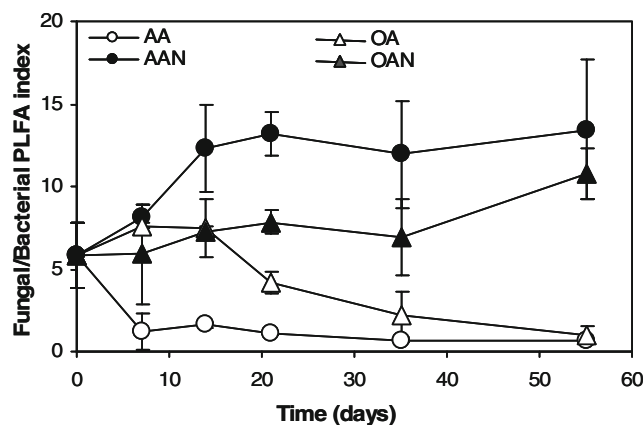


Fig. 2 Time course of the change in fungal/bacterial PLFA index. The index was calculated as the ratio between fungal (18:2 ω 6.9) and bacterial (i15:0, a15:0, i16:0, i17:0, cy17:0, 18:1 ω 7c and cy19:0) fingerprints abundances. Each data point corresponds to the mean value for triplicate reactors (bars correspond to \pm standard deviation). Symbols refer to the different treatments: aerated (AA), aerated + nutrients (AAN), non-aerated (OA) and non-aerated + nutrients (OAN)

2. *TTGE analysis.* Overall, the results of the comparative TTGE analyses revealed significant differences in bacterial and fungal communities when varying nutrient addition and aeration to the bioreactors (Figs. 3 and 4). These differences between the treatments were clear from the first week of the experiment (day 7), i.e., there was rapid establishment of the different microbial communities under all the conditions of nutrient status and aeration. Thereafter, the communities were stable, since their individual TTGE profiles did not change during the remaining 48 days of the experiment.

The greatest bacterial diversity, in terms of number of discrete bands, was detectable in OAN bioreactors (14–17 bands per sample), followed by AA (6–15 bands), AAN (6–10 bands) and finally by OA (4–5 bands). The TTGE profiles showed that the greatest fungal diversity was detected in OAN bioreactors (14–17 bands), followed by AAN (10–13 bands) and AA (7–9 bands). Thus, the complexity of the microbial communities did not seem to be linked with the degradation of polyphenols. For instance, the maximal polyphenol degradation was detected in AAN bioreactors, but the most complex communities, in terms of number of TTGE bands, was found in OAN bioreactors. Therefore, these results show that only a few specific microorganisms, which were stimulated with nutrients and oxygen supply, were responsible for the greatest degradation of polyphenols.

As expected, in both bacterial and fungal TTGE analyses, samples from the same treatment clustered together (Fig. 5). No PCR product was obtained from any of the OA samples using fungi primers. In the cluster analysis of bacterial TTGE, two major groups could be observed, one corresponding to the AAN bioreactors and the other to the rest of the samples. In contrast, in the fungal TTGE analyses, profiles from the OAN treatment clustered

apart from profiles of aerated bioreactors. Moreover, TTGE profiles seemed to be independent of the incubation time. It is important to note that, despite the high similarity of profiles that belonged to the same treatment (for bacteria and fungi analysis), each lane of the TTGE gels was obtained from DNA isolated from a different (independent) bioreactor.

To perform sequence-based phylogenetic identification, a sequence analysis of specific bands was performed. Twenty newly determined sequences were compared directly with those in GenBank using BlastN. Database similarity is shown in Table 1. A total of 14 different genera of bacteria were detected, belonging to Gammaproteobacteria (5), Actinobacteria (4), and Firmicutes (5). Only two sequences were obtained from the samples of the initial mixture (day 0), both corresponding to the genus *Bacillus*. In fungal TTGE gels, a total of 14 sequences were successfully obtained (Table 2). BLAST analysis confirmed the specificity of the primers used, as all sequences could be affiliated to fungal genera in more than 97% similarity. In total, 6 genera were identified, all of them belonging to *Ascomycota*, namely *Penicillium*, *Candida*, *Geotrichum*, *Pichia*, *Cladosporium*, and *Aschochyta*.

Discussion

The majority of previous studies dealing with the biodegradation of olive wastes have involved sterilization of the residues before inoculation of a specific microorganism or consortium. However, not only does autoclaving kill the indigenous microbiota, but also the high temperature causes significant physico-chemical modifications such as the oxidation of aromatic compounds present in the olive wastes (Aggelis et al. 2003). A simple, unmodified system was selected in this study since a major aim was to provide

Fig. 3 PCR-TTGE profiles of 16S rRNA fragments representing the bacterial diversity of species generated from the samples taken at days 0, 7, 14, 21, 35, and 55. The bands identified by sequencing are indicated by the arrowheads. Bioreactor treatments: aerated (AA), aerated + nutrients (AAN), non-aerated (OA) and non-aerated + nutrients (OAN)

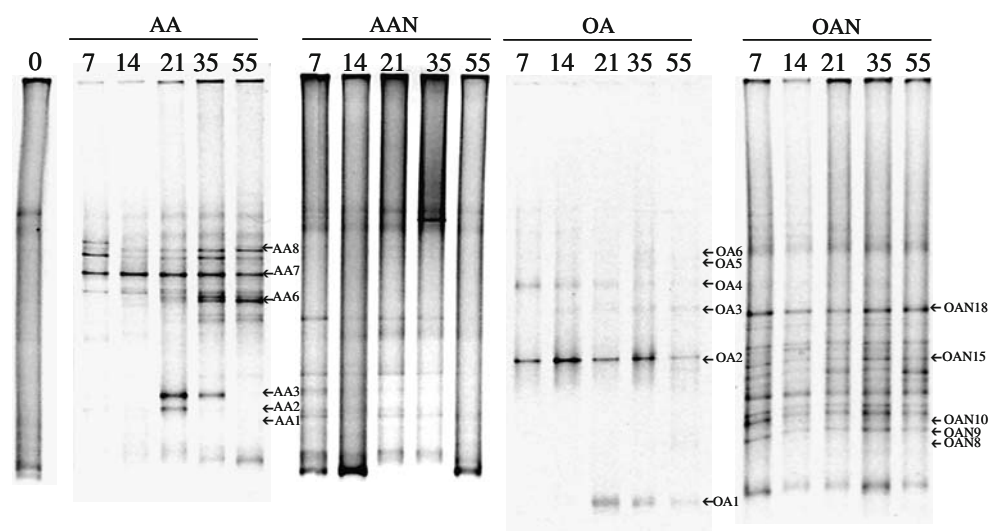
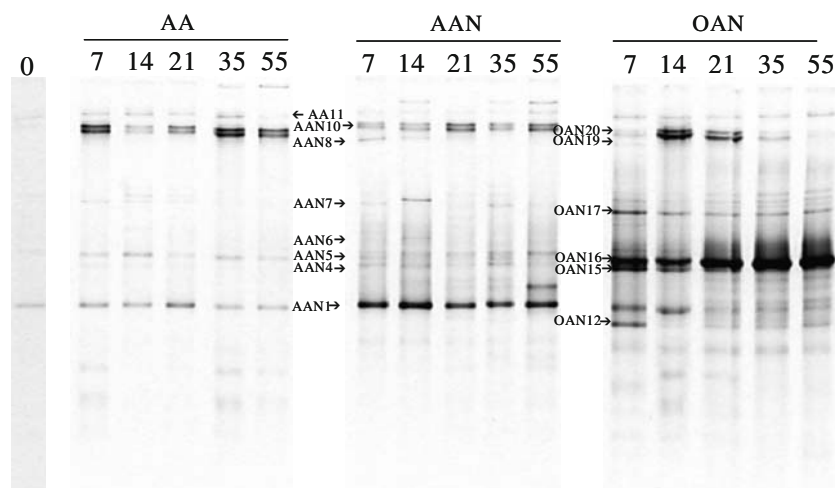


Fig. 4 PCR-TTGE profiles of ITS region fragments representing the fungal diversity of species generated from samples taken at days 0, 7, 14, 21, 35, 55. The bands identified by sequencing are indicated by the arrowheads. Bioreactor treatments: aerated (AA), aerated + nutrients (AAN), non-aerated (OA) and non-aerated + nutrients (OAN)



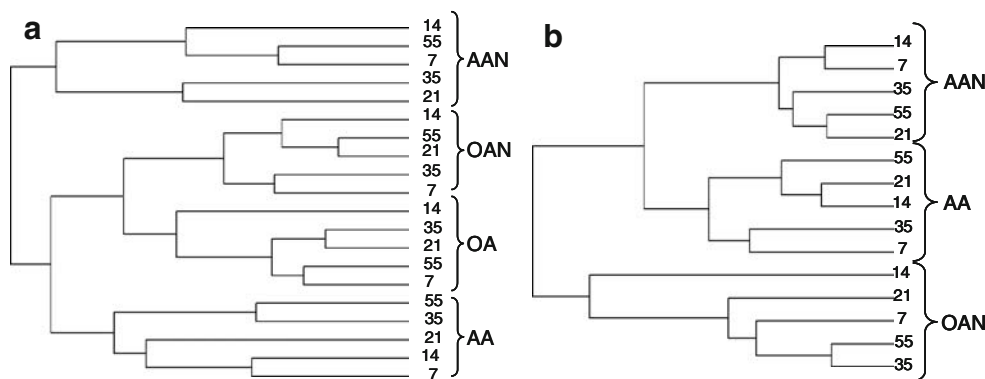
fundamental information that is relevant to the development of a robust biotechnological application for eventual optimized scale-up to industrial scale. Therefore, in the work presented here, the TPOMW was not sterilized or manipulated in any other way before preparing the (bio) degradation mixture, adding only double distilled water and wheat straw to improve the porosity of the material and thus enabling bioremediation to occur. Wheat straw may have contributed to the mixture with some microbial activity, but given the organic richness of the TPOMW, it is most likely that the bulk of the microbiota were derived from the original olive waste. Therefore, it was assumed that the genetic potential of the indigenous microbiota of the waste (olive waste plus wheat straw) is able under favourable conditions to metabolize polyphenolic compounds present in the mixture. It is here suggested that the stimulation of this indigenous microbiota is a promising alternative bioremediation approach. Compared with the inoculation of a single strain (or consortium) approach, indigenous microorganisms have a range of different biodegrading activities that could act simultaneously or sequentially.

For the purpose of this study, a temperature of 25°C was selected, rather than a higher value, as being one that could

be readily achieved in an industrial scale development without the necessity for expensive energy input. Break-down of polyphenols occurred mainly during the first 7 days of incubation, and organic matter losses occurred mainly during the first 14 days (Fig. 1). Thus, during the initial 1–2 weeks incubation period, a microbial community with the capacity to metabolize polyphenolic compounds present in TPOMW was established in all bioreactors, except the aerated non-amended ones. Increases in polyphenol concentration (mg g^{-1} dry matter) during treatment were occasionally observed in the reactors (Fig. 1a). The experimental variation of moisture content or flow rate during the treatment would potentially affect the polyphenol biodegradation extent and rate in the mixtures, although they would not explain an increase in polyphenol concentration. Thus, an occasional increase in polyphenol concentration might be the consequence of a selective biodegradation of organic matter within the TPOMW and straw mixture, changing the ratio of TPOMW to wheat straw in the mixture and therefore in the calculation of the concentration of polyphenols in the mixture as previously observed (Antizar-Ladislao et al. 2005).

Although PLFA analysis detected changes in the relative proportions of bacteria and fungi, and of Gram-positive and

Fig. 5 Cluster analysis of TTGE band patterns for bacteria (a), and fungi (b). The numbers indicate the day of sampling. Bioreactor treatments: aerated (AA), aerated + nutrients (AAN), non-aerated (OA) and non-aerated + nutrients (OAN)



Gram-negative bacteria, TTGE profiles showed that microbial community structures were established rapidly, within 7 days, and that thereafter, the predominant microbial species in the bioreactors were stable throughout the incubation period, i.e., not only during the initial phases of maximum polyphenol biodegradation and TOM decrease, but also during the later stages when less biodegradation occurred. Partial rDNA bacterial and fungal sequence comparisons with sequences in the database gave values that ranged from 84 to 99% and from 92 to 100%, respectively, although most values were higher than 97%. One should notice that these values could differ if complete sequences were compared. Most of the bacteria, as identified by TTGE, which are most likely to comprise the predominant members of the microbial community in TPOMW-wheat straw mixtures, are phylogenetically associated with bacteria, such as *Luteibacter* sp., that have previously been isolated from plant material (Johansen et al. 2005). Additionally, some have been described as plant pathogens or at least associated with plant diseases, such as *Rhodococcus fascians* and *Clavibacter michiganensis*, present in the bioreactors and known to be plant pathogens (Young et al. 1996). Furthermore, *Stenotrophomonas maltophilia* is another plant pathogen that is able to degrade polyphenolic compounds (Franco et al. 2005). Actinomycetes belonging to the genus *Curtobacterium* have also been isolated from plant material (Postma et al. 2005). *Clostridium saccharolyticum*, which was isolated from sewage sludge (Murray et al. 1982), and *C. xylanolyticum* (Rogers and Baecker 1991) are both able to biodegrade polymers that could have a role in the bioremediation of TPOMW. The finding of anaerobes such as clostridia in aerated bioreactors could be regarded as an anomaly, but as the material in the bioreactors is very heterogeneous, it is possible that there were local anaerobic “pockets” within the organic material that could harbor clostridia which are able to tolerate the intermittent presence of oxygen.

The greatest biodegradation of polyphenols occurred in those bioreactors that were both nutrient amended and aerated. These reactors presented higher fungal to bacterial biomass ratios, as indicated by PLFA analyses. Fungi have previously been studied for their capacity to remove polyphenols from olive residues by the release of extracellular enzymes, particularly lignin peroxidases, Mn-dependent peroxidases and laccases, which not only biodegrade polyphenols but also decolorize olive-waste residues (Assas et al. 2000; Linares et al. 2003). Yet, the rate and extent of the removal of polyphenols depends on the particular fungi present and also on the nature of the polyphenols (Sampedro et al. 2004). This study reports for the first time a molecular biological investigation of fungal communities involved in the process of olive mill waste bioremediation.

Strains belonging to *Penicillium*, *Geotrichum*, *Pichia*, and *Candida* were the major fungi detected by TTGE. These genera have previously been isolated from different OMWs including TPOMW (Assas et al. 2000; Ettayebi et al. 2003; Millan et al. 2000), demonstrating that they are likely to be part of the natural microbiota of these residues. Some of these fungi show a promising range of biotechnological applications in connection with the treatment of OMWs (D’Annibale et al. 2005; Ayed et al. 2005).

In conclusion, this study highlights that probably a relatively small number of strains within the indigenous microbiota of the TPOMW is able to biodegrade the polyphenolic content of the waste without the inoculation of specific microorganisms. The results presented here indicate that the fungal community should be stimulated by the addition of nutrients and air for improved polyphenol biodegradation. However, the contribution of the bacteria able to grow in this waste should be considered. Further research is necessary to optimize the conditions for improved bioremediation of olive waste, and different biotechnological applications could be coupled to the process.

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