Chestnut Biomass Biodegradation for Sustainable Agriculture

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Biodegradation of lignocellulosic waste from chestnut residues is an important biological process of added value for this sector according to the Chestnut National Plan for agriculture. Dynamic parameters during biodegradation under natural conditions of chestnut burr, leaf, and plant biomass litter are reported in this study. Microbiological and physical-chemical characterisation of chestnut wastes was carried out to monitor this specific biodegradation, to understand the progress and limits of the process and to analyse the compost-like organic substance obtained. Physical-chemical parameters, such as temperature, pH, and water activity, were influenced by the composition of the raw materials and by seasonal climatic conditions. Moreover, microbiological monitoring was assessed by culture-dependent and independent methods. Cellulolytic, hemicellulolytic, and ligninolytic populations were counted to determine different microbial activity during biodegradation process. The functional microbial groups analysed showed different trends, but all were found at high concentrations (7 to 9 log CFU/g). In addition, PCR-DGGE was performed for bacterial and fungal populations to evaluate the microbial diversity. The similarity level during the process was generally very high, both for bacterial and fungal populations. These data are the first on suitable natural degradation for chestnut forests.

Keywords: Chestnut composting; Microbiological monitoring; PCR-DGGE; Sustainable fertilisation

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INTRODUCTION

The Italian Chestnut National Plan in Italy supports the need to guide the chestnut population toward more sustainable forms of production, characterised by a higher ability to meet the needs and interests of consumers and producers by offering new economic opportunities in agriculture. These measures are justified by the need to sequester carbon, reducing greenhouse gas emissions into the atmosphere, recovering biomass for agronomic purposes, and increasing the sustainability of agricultural productions (Marmo 2007).

The use of biomass is interesting at both the EU and national level, especially after the agreements of the Kyoto Protocol (reduction of CO_2 emissions into the atmosphere). Recently, on-farm composting processes have been developed to transform biomass waste of agricultural origin (Tuomela *et al.* 2000). The use of lignocellulosic materials in composting has several important roles, such as structural, absorptive, and biochemical changes (Hubbe *et al.* 2010). An interesting example is the composting of lignocellulosic winery wastes (Paradelo *et al.* 2013*)*. The evaluation of compost made from chestnut residues obtained from cleaning and pruning can be a source of added value for the sector. Although composting procedures are well known, very few reports

exist on the biodegradation of chestnut wood remains and their use in agriculture, and these residues are often burned (Guerra-Rodrìguez *et al.* 2006). Chestnut residue composting, related practices, and their utilisation in agriculture would be possible approaches to a sustainable management of chestnut forests. Benefits of application of biodegraded matter are well recognised. The use of compost, for instance, contributes to the following: (1) improving the soil structural stability, increasing its fertility, and recycling organic matter (humus) and nutrients in a slow release; (2) restoring microbiological diversity in soil and limiting the development of plant pathogens and plant diseases; (3) increasing biological activity and root aeration; (4) reducing the environmental impact, contributing to the degradation of pollutants, and increasing crop protection and carbon dioxide storage in soils; and (5) reducing the use of chemicals and disposal costs.

Moreover, a further relevant aspect involves mechanisms by which compost's microflora are able to contain pathogens. This consists of competition between pathogens and antagonists, antibiosis, parasitism, and induction resistance in the host plant (Hoitink *et al*. 2001). Particularly interesting is the use of compost to suppress soil-borne pathogens. This aspect has been extensively studied by several authors (De Ceuster and Hoitink 1999; Pointing 1999; Ryckeboer *et al*. 2003). The studies of biotic and agronomic components represent key points to characterise the compost (Pepe *et al*. 2013a).

Many studies have been conducted in pilot-scale reactors that enable easier tracking of the biological process: field experiments do not allow monitoring developments in biodegradation because it depends on many environmental factors. Static aerated heaps have been developed for the purpose of natural degradation of lignocellulosic waste obtained from the cleaning of chestnut. Microbiological monitoring using culture-dependent and culture-independent methods of physical-chemical parameters such as temperature, pH, and water activity have been carried out during the process of biodegradation under natural conditions. In fact, the efficiency of process stages depends on a variety of parameters, including aeration, temperature, and content of moisture in the waste. The most important aspect, however, is represented by the microorganisms involved and their functional activity (Neklyudov *et al*. 2008; Pepe *et al*. 2013a).

The present study aims to create useful innovations to increase the sustainability and profitability of production, in accordance with the regulations of organic farming and environmentally-friendly criteria. Biotechnological and agronomic innovations will contribute to the well-being of those who work in remote areas, creating less impact on the environment and improving the quality of the product. On the basis of these considerations, microbiological and physical-chemical characterisation of chestnut biomass submitted to biodegradation was carried out to investigate the bio-oxidative processes at different times and analyse the compost-like organic substance obtained from a microbial and agronomic perspective to understand the progress and limits of the process.

EXPERIMENTAL

Characteristics of the Lignocellulosic Waste

The experimental field was in Roccamonfina (province of Caserta), an area in southern Italy particularly suitable for chestnut forests. The compost-like organic substance (CLOS) was obtained from a composting bin, and the resulting materials were placed in piles. The size of the bin was 1.50 m x 1.50 m x 1.80 m (length x width x height). Fagots were placed under the bin to avoid water retention. The structure was filled with the resulting material of chestnut cleaning (without shredding), such as fresh shoots, chipboard obtained from the cleaning of stakes, undergrowth vegetation (ferns, vetch, sainfoin, wild oats, horsetail grass, and calenzuola), old leaves, and curly. All components were added in equal amounts in terms of weight (about 60 kg) and submitted to natural degradation. Finally, the vegetable biomass was covered with 5 cm of soil taken from the chestnut forest to create a sort of natural lid, which can also enhance the biodegradation process.

The degradation of lignocellulosic wastes was carried out in natural conditions for 105 days. During the whole process, the pile was never turned. Moreover, aeration was also ensured by gaps formed by lateral and basal fagots.

Sampling

Since the process was submitted to natural degradation, it was assumed that it was slow and therefore the samples were collected every two weeks. Samples of 1 kg were collected from the external (right and left side of the pile) and from the internal central part of the biomass and mixed before analyses. Sampling was performed immediately after preparation (T0) and at 15, 30, 45, 60, 75, 90, and 105 days (T1, T2, T3, T4, T5, T6, and T7, respectively) of biodegradation.

Physical-chemical Monitoring and Microbiological Count

CLOS samples were monitored using physical-chemical and microbiological parameters at T1, T2, T5, T6, and T7.

Temperature (°C) was monitored using specific sensors (1500 mm) placed in the core of the pile. The pH was also measured by resuspending 25 g of sample in 250 mL Ringer's solution. Finally, water activity (a_w) was evaluated using a HygroPalm23-AW (Rotronic AG, Basserdorf, Germany). C/N ratio was determined based on the concentration of carbon (Nelson and Sommers 1996) and of nitrogen value (Stevenson 1996).

For microbiological counting, an initial suspension was prepared by the addition of 25 g (wt/vol) of the samples to 180 mL of quarter-strength Ringer's solution (Oxoid, Milan, Italy) in 225-mL Erlenmeyer flasks. After shaking, suitable dilutions were made to the sample solution, which was then used to inoculate different solid growth media. Specific functional groups of the soil microbial community involved in the carbon cycle, such as cellulolytic, hemicellulolytic, and ligninolytic populations, were detected at 28 °C using the surface spread plate count method. Cellulolytic microorganisms were counted using a minimal medium (1 g L^{-1} $(NH_4)NO_3$, 1 g L^{-1} yeast extract, 50 mL L^{-1} standard salt solution, 1 mL L^{-1} trace elements solution, and 15 g L^{-1} bacteriological agar, at pH 7.0) with carboxymethylcellulose (5 g L^{-1}) as the sole carbon source and Remazol Brilliant Blue R to show evidence of cellulolytic activities by developing clear haloes around the colonies (Ventorino *et al*. 2010). The medium used for hemicellulolytic population determination was the minimal medium described above with xylan (5 g L^{-1}) as the sole

carbon source. Finally, ligninolytic microorganisms were counted using LME basal medium (1 g L^{-1} KH_2PO_4, 0.5 g L^{-1}, $C_4H_{12}N_2O_6$, 0.5 g L^{-1} $MgSO_4$, 0.01 g L^{-1} $CaCl_2$, 0.01 g L^{-1} yeast extract, 0.001 g L^{-1} $CuSO_4$, 0.001 g L^{-1} $Fe_2(SO_4)_3$, 0.001 g L^{-1} $MnSO_4$, and 16 g L^{-1} bacteriological agar) supplemented with 0.25 % (wt/vol) lignin as the carbon source, as described by Pointing (1999).

DNA Extraction

Microbiological monitoring with a culture-independent method was performed for all samples (T0, T1, T2, T3, T4, T5, T6, and T7).

Microbial cells were desorbed from CLOS matrices, and pellets were obtained prior to community DNA extraction, as described by Rosewarne *et al*. (2011) with some modifications. First, 100 g of the samples was resuspended in 400 mL of acidic solution (pH 2.0), shaken at 240 rpm for 10 min, and filtered and centrifuged at 10,000 rpm for 20 min. Microbial cells were resuspended in 45 mL of sterile phosphate buffered saline (PBS), then recovered by centrifugation at 6500 rpm for 20 min. The DNA was extracted from compost samples using the FastDNA Spin Kit for Soil (MP Biomedicals) according to the supplier's recommendation.

PCR-DGGE

The primers V3f (5'-CCTACGGGAGGCAGCAG-3') and V3r (5'-ATTACC GCGGCTGCTGG -3') spanning the 200-bp V3 region of the 16S rDNA of *E. coli* were used for bacterial PCR-DGGE analysis. The PCR mixture and conditions were as previously described (Pepe *et al*. 2013b). To analyse the yeast population, the primers NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and LS2 (5'-ATTCCCAAACAA-CTCGACTC-3') corresponding to nucleotide positions 266 to 285 on the *S. cerevisiae* 26S rRNA gene were used. The PCR mixture and conditions were as previously described (Palomba *et al*. 2011). A GC-clamp was added to the forward primer in all PCR reactions, following Muyzer *et al*. (1993).

DGGE analyses were performed using a Bio-Rad DCode Universal Mutation System (Bio-Rad Laboratories). PCR products were loaded in a 0.8-mm polyacrylamide gel [8% (wt/vol) acrylamide-bisacrylamide (37:5:1)] using a denaturant gradient from 30 to 60% increasing in the direction of electrophoresis for both bacterial and fungal analysis. Electrophoresis was performed at 60 °C, initially at 50 V (5 min) and then at 200 V (240 min). The gels were stained in an ethidium bromide solution (5 min) and rinsed in distilled water (20 min).

Statistical analysis

Bands were automatically detected using the software Phoretix 1 advanced version 3.01 (Phoretix International Limited, Newcastle upon Tyne, England). The cluster analysis was performed by the program after band matching; the method described by Saitou and Nei (1987) was used to obtain the correlation matrix of the DGGE patterns. The resulting matrix was used in the average linkage method by the Cluster procedure of Systat 5.2.1 to estimate the percentage of similarity (S) of microbial populations in the composting process.

RESULTS AND DISCUSSION

The site used for setting up the experiment was chosen because its climatic characteristics (400 m above sea level and north-west sun exposure) are representative of a chestnut forest. The biodegradation bin was filled with the resulting material of the chestnut cleaning, as shown in Fig. 1.

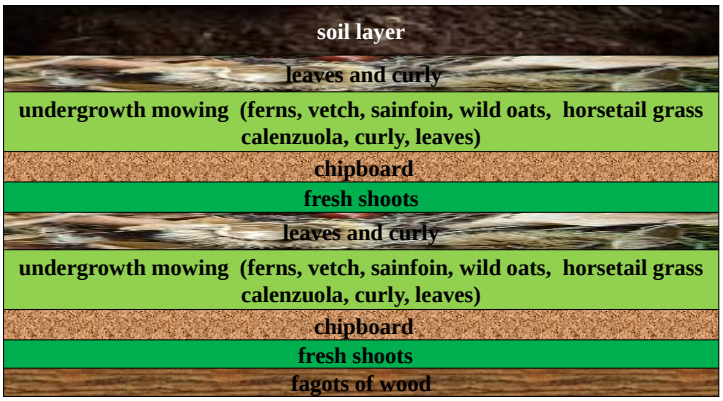

Fig. 1. Order and composition of layers in the biodegradation bin

The biochemical composition of the raw materials was: UR 40.2%, pH 4.54, organic carbon 28.9 % (dry weight), total nitrogen 0.5 % (dry weight), ammonia nitrogen 0.45 % (dry weight), C/N ratio 57.8.

The periodic measurement of the temperature during the biodegradation process is an important index to monitor the fermentation process because it is closely related to environmental conditions and to the metabolic activities of microorganisms. After 15 d, the temperature value observed in the pile was 27.1±0.06 °C (T1 sample). This value increased to 28.9±0.06 °C after 90 days of biodegradation (T6 sample). Since the biodegradation process was not carried out in controlled conditions but in a chestnut forest, it might be inferred that the temperature values in the internal part of the pile were strongly affected by the seasonal climatic conditions. In fact, as shown in Fig. 2, the temperature increased until September (28.9±0.06 °C) and underwent a sudden fall in October (26.6±0.07 °C). The detected environmental temperature showed a similar trend (Fig. 2). Although there are no common definitions of mesophilic and thermophilic phases during the composting process, mesophilic generally refers to temperatures up to approximately 40 °C, and thermophilic refers to temperatures from 45 to 70 °C (Miller 1996). The temperature values detected in this work suggest that the natural degradation process was in the mesophilic phase after 105 days.

Moreover, the detected carbon/nitrogen ratio of raw materials was very high (57.8) to reach a complete degradation. Guerra-Rodríguez *et al*. (2001a) reported that the composting of chestnut burr and leaf litter did not occur because the C/N ratio was too high and the compost never reached the thermophilic phase. In contrast, co-composting with solid poultry manure reached the temperature needed for the thermophilic phase of the process (about 60 °C) in 14 days (Guerra-Rodríguez *et al*. 2001a).

Moreover, measurements of other parameters that influenced microbial activity, such as pH and water activity (a_w), were monitored to understand the progress and limits of the process.

The pH values observed were quite stable, ranging from 6.75±0.03 to 6.8±0.02 at T1 and T7, respectively (Fig. 3). The biodegradation process should occur at a pH value from 3 to 11 (Guerra-Rodríguez *et al*. 2001b), but the best results are obtained in the range of 5 to 8 (Sundberg *et al*. 2004). Beck-Friis *et al*. (2001) reported that the change from the mesophilic to the thermophilic phase in composting corresponded to a change in pH from a range of 4.5 to 5.5 to a range of 8 to 9. In fact, in the first phase of the process, the acidic pH is due to the production of organic acid by microorganisms (Guerra-Rodríguez *et al*. 2001b), while the increase in pH up to 9 is due to ammonium production from protein degradation (Mahimaraja *et al*. 1994).

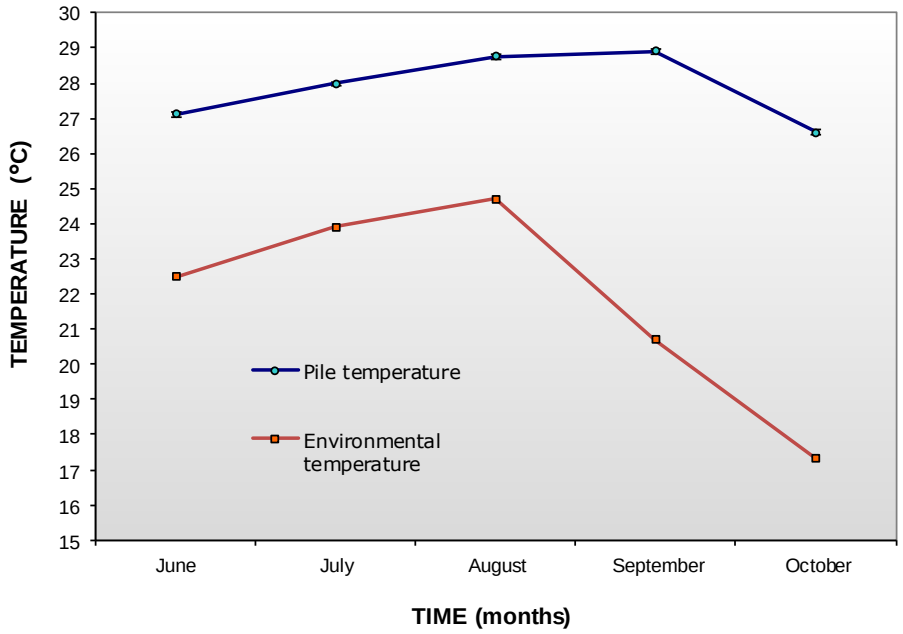

Fig. 2. Temperature values determined during the natural degradation process

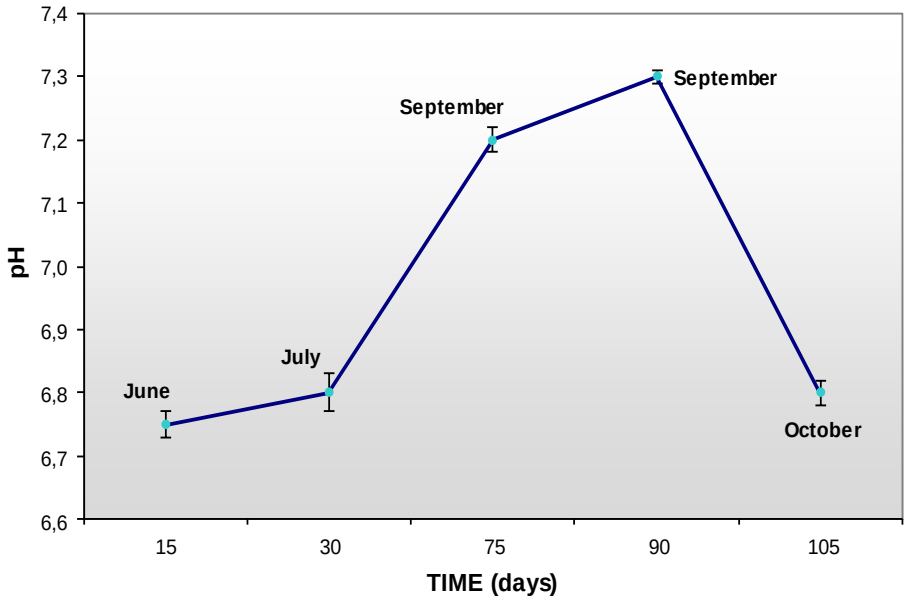

Fig. 3. pH values determined during the natural degradation process

Water activity (a_w) (water readily available for microbial metabolic activities) was also measured. The value of a_w was 0.94±0.003 after 15 d of experimentation and increased up to 0.98±0.001 after 105 days of the natural degradation process (Fig. 4).

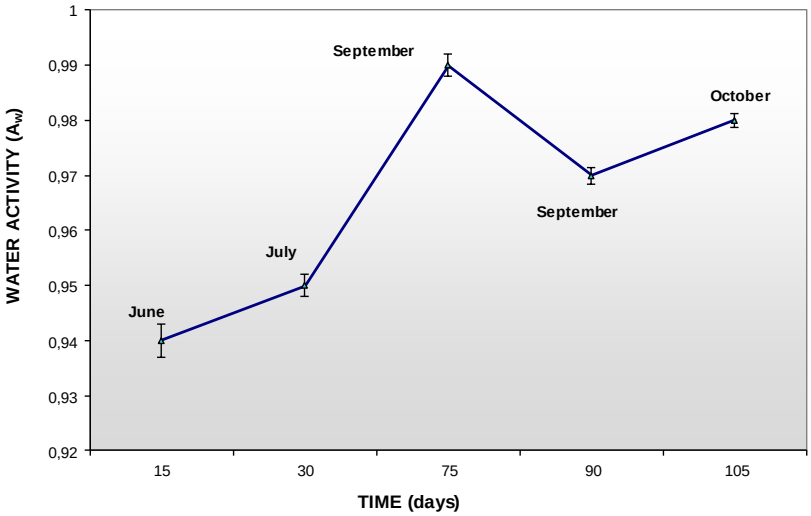

Fig. 4. Water activity values determined during the natural degradation process

Biodegradation of lignocellulosic materials is a result of the combined and sequential action of specific functional microbial groups, but little is known about the diversity of microorganisms in the carbon cycle that is specific and essential for vegetable biomass degradation. A large number of microorganisms have been studied for their potential ability of cellulose, hemicellulose, and lignin degradation in different experimental conditions (Huang *et al.* 2010a). The effectiveness of the process depends largely on the metabolic activities of microorganisms affecting the quality of the end product. For this reason, knowledge of the importance of specific functional groups can still be improved (Pepe *et al.* 2013a).

Because lignocellulosic materials are complex molecules of hemicellulose, cellulose, and lignin, three different functional microbial groups were analysed. The three functional groups involved in the carbon cycle showed different trends during biodegradation under natural conditions. Hemicellulolytic populations showed a value of about 9 log CFU g^{-1} of sample during the experimental process (Table 1). Indeed, after 105 days of biodegradation (T7), it was possible to detect an increase in cellulolytic populations, showing a concentration reaching to 10.18±0.04 log CFU g^{-1}. Observing the growth of aerobic cellulolytics, it was thought that the organic matrix possibly showed a good starting composition of cellulosic material. Pepe *et al.* (2013a) observed an increase in aerobic cellulolytics growth during composting of agro-industrial waste. The presence of hemicellulolytics and cellulolytics during the whole biodegradation process plays an important role with respect to cellulose, which represents the principal constituent of vegetable waste and whose degradation is restricted to a narrow range of microbial enzymes such as hemicellulase and cellulase (Herrmann and Shann 1997; Amore *et al.* 2013). Ligninolytic microorganisms showed an opposing trend. For this population, a significant decrease of about 1 log unit (from 8.48±0.00 to 7.59±0.16 log CFU g^{-1}) was detected (Table 1). It is probable that the decrease of temperature at T7 could negatively influence ligninolytic microbial activities, especially with regard to the fungal population.

In fact, microorganisms usually involved in the degradation of structural polymers (cellulose, hemicellulose, and lignin) such as fungi and actinomycetes (Huang *et al.* 2008; Amore *et al.* 2012) could be negatively affected by low temperature because their growth is favoured by higher temperatures (Ishii *et al.* 2000). Huang and co-workers (2010b) reported a continuous change in microbial population structure during composting of lignocellulosic waste. In particular after 3 days they detected a decrease of quinone species Q-9 associated with lignin degraders and an increase of Q-9(H2) and Q-10(H2) associated with cellulose-degrading ability.

Table 1. Microbial Contents (Log CFU g^{-1}) During Biodegradation Process

TIME SAMPLING (days)*	MICROBIAL FUNCTIONAL GROUP		
	Hemicellulolytic	*Cellulolytic*	*Ligninolytic*
15	9.04±0.06	9.00±0.06	8.48±0.00
75	9.36±0.08	9.65±0.07	9.25±0.07
90	8.58±0.03	8.64±0.19	7.34±0.19
105	8.97±0.21	10.18±0.04	7.59±0.16
* 15, T1; 75, T5; 90, T6; 105, T7.			

Microbiological monitoring with a culture-independent method was carried out during the natural degradation process. The results of the PCR-DGGE analysis performed on the samples during biodegradation are shown in Fig. 5. After 15 d, the bacterial and fungal populations showed very different profiles compared to the beginning of the process (T0). Instead, during natural degradation, the microbial community varied slightly, showing similar profiles both in bacterial and fungal populations. Ahmad *et al*. (2011) reported that the microbial community was influenced by environmental conditions and substrate characteristics, but did not change significantly during a composting process. In fact, they reported that the DGGE analysis showed similar profiles and the presence of same major bands.

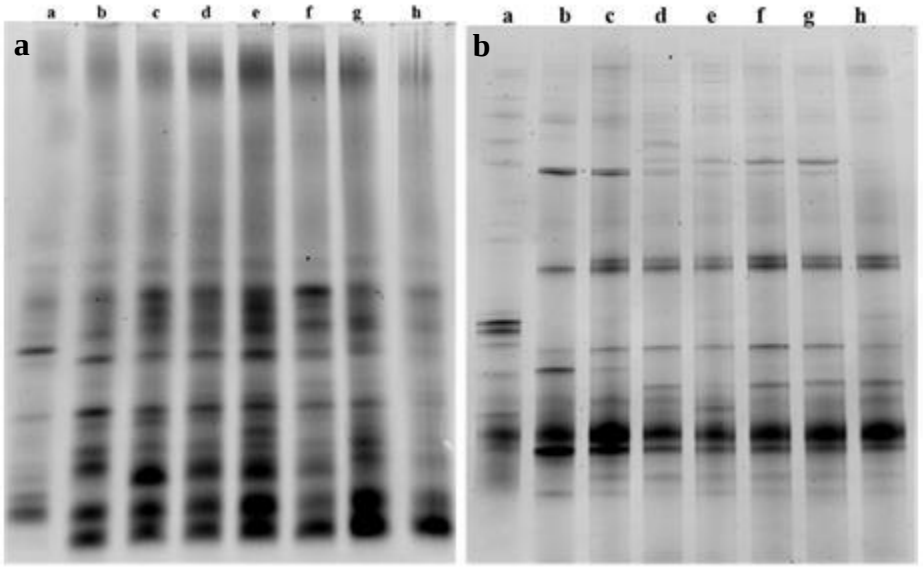

Fig. 5. DGGE profiles of bacterial (a) and fungal (b) populations during biodegradation process. Lanes: a, T0 (0 d); b, T1 (15 d); c, T2 (30 d); d, T3 (45 d); e, T4 (60 d); f, T5 (75 d); g, T6 (90 d); h, T7 (105 d)

The similarity level (S) in bacterial populations during chestnut biomass biodegradation was generally very high, showing values from 95% to 100%, except for at T0 (Fig. 6a). In fact, only at the beginning of the process were the bacterial populations very different, showing a similarity value of 60%, unlike the other sampling times. A similar trend was observed in fungal populations (Fig. 6b). In this case, the microbial diversity detected at T0 was the highest, showing a similarity value of 42% with the other times. Moreover, the levels of similarity among the fungal populations during the natural degradation varied more gradually than those detected among bacterial populations, as shown in Fig. 6.

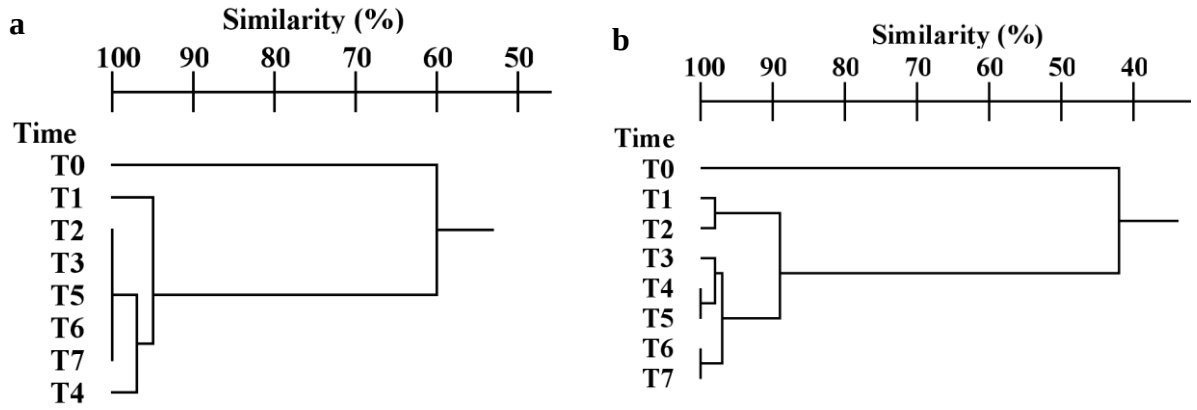

Fig. 6. Dendrogram showing the degree of similarity (%) of PCR-DGGE profiles of the bacterial (a) and fungal (b) populations during natural degradation of chestnut biomass

These results showed that the bacterial population was less influenced by the environmental conditions than the cellulolytic microbial functional group. In contrast, the fungal population suffered a greater variation during the biodegradation process, showing behaviour similar to a ligninolytic functional group. Because the ligninolytic activity of fungal populations is widely recognised (Feng *et al.* 2011), it is possible that the ligninolytic group was composed mainly of fungi and less so of bacteria, which exhibit more cellulolytic and hemicellulolytic activities. Despite recent findings, our results are encouraging because there have been few reports on biodegradation of chestnut biomass and there are no previous investigations about its microbiological characterisation.

CONCLUSIONS

1. The temperature values in the internal part of the pile were strongly affected by the seasonal climatic conditions.
2. Cellulolytics increased during biodegradation of chestnut biomass, while ligninolytics decreased as an effect of microbial competition and environmental conditions.
3. DGGE analysis showed similar profiles during the natural degradation process for both bacterial and fungal populations, except at the beginning of the process.
4. Because lignocellulosic biomass is also an attractive waste material for production of a wide range of high value-added products, cellulolytic, hemicellulolytic, and lignin-

lytic microorganisms should be isolated and selected for their biotechnological applications and used to improve the process.

5. To the authors' knowledge, there have been no previous investigations of the microbiological characterisation of chestnut biomass submitted to biodegradation under natural conditions. The first preliminary results are encouraging, but further tests must be carried out to improve the understanding of natural degradation of chestnut and the final product.

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