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Microbially-enhanced composting of olive mill solid waste (wet husk): Bacterial and fungal community dynamics at industrial pilot and farm level

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HIGHLIGHTS

- Community dynamics analysed by DGGE along composting wet husk at pilot and farm level.
- Starters enhanced transformation and increased earlier bacterial diversity.
- High diversity in thermic phase was a typical trait of wet husk transformation.

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ABSTRACT

Bacterial and fungal community dynamics during microbially-enhanced composting of olive mill solid waste (wet husk), used as a sole raw material, were analysed in a process carried out at industrial pilot and at farm level by the PCR-DGGE profiling of the 16 and 26S rRNA genes. The use of microbial starters enhanced the biotransformation process leading to an earlier and increased level of bacterial diversity. The bacterial community showed a change within 15 days during the first phases of composting. Without microbial starters bacterial biodiversity increased within 60 days. Moreover, the thermophilic phase was characterized by the highest bacterial biodiversity. By contrast, the biodiversity of fungal communities in the piles composted with the starters decreased during the thermophilic phase. The biodiversity of the microbial populations, along with physico-chemical traits, evolved similarly at industrial pilot and farm level, showing different maturation times.

1. Introduction

Composting and co-composting of olive mill solid waste (wet husk) is receiving an increasing attention (Alfano et al., 2008; Hachicha et al., 2009), and the resulting compost has recently been shown to improve nutraceutical traits of horticultural crops (Ulrichs et al., 2008) and to represent a fertilizer for short-term crops (Altieri and Esposito, 2010). Detoxification of fats, organic acids and polyphenols is achieved throughout the process, resulting in an odourless product with a good germination and humification index (Echeverria et al., 2011). These results are due to the biotransformation activity of microorganisms, leading to a rapid succession of specialized bacterial populations during co-composting (Federici et al., 2011). Various attempts have been carried out to identify the microbial species and to enumerate the physiotaxonomical groups (bacteria and fungi) during the three phases of husk composting or co-composting, namely the activation, thermophilic, and maturation phase, using cultivation-dependent methods. However, the results are erratic and provide underestimates due to the constraints of culture media and cultivation conditions (Principi et al., 2003; Bru-Adan et al., 2009), as well as to the presence of microbial communities in viable but non-culturable state.

Culture-independent approaches are becoming prominent to study microbial communities structure and dynamics, and molecular methods such as PCR-DGGE have been used to analyze microbial biodiversity during the composting process of different matrices (Novinscak et al., 2009; Takaku et al., 2006; Zhang et al., 2011). According to such studies, the microbial communities are highly variable during the various phases of the composting process and tend to get stabilized at compost maturity. This approach has been recently used by Vivas et al. (2009) to analyze the bacterial community structure in the final vs. in the initial matrix of a mixture of fresh olive waste and sheep manure processed by co-composting or vermicomposting. They found that the bacterial diversity was markedly affected by vermicomposting but not by co-composting. The use of starter cultures to speed up the

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composting process or to obtain improved compost has been controversial for long time, probably due to the complexity of the physical–chemical and biological events occurring during the process (Vargas-Garcia et al., 2006). Indeed, the selection of appropriate microbial strains can represent a valid alternative to the traditional treatment of wet husk, as it improves both biotransformation speed and the quality traits of the final product (Echeverria et al., 2012).

This study aims at profiling the fungal and bacterial communities during a composting process of olive mill solid waste (wet husk) as a sole raw material enhanced by the use of microbial starters at industrial pilot (representing the best biotransformation scenario) and at farm level (with limited process control facilities, thus representing the worst case scenario for the disposal and upgrade of wet husk through composting). The variability and the diversity of the microbial community were estimated by UPGMA analysis of the PCR-DGGE profiles and by the community diversity indices analysis.

2. Methods

2.1. Composting procedure, sampling and physical–chemical analyses

The microbial starters used throughout this study were *Bacillus amyloliquefaciens* subsp. *plantarum* M51/II [formerly *Bacillus subtilis*, reclassified according to Chen et al. (2009) and Borriss et al. (2011)], *Pseudomonas synxanta* 3/2, *Pseudomonas fluorescens* 19/5, *Serratia marcescens* B2 (bacteria), *Streptomyces* sp. ATB 42, *Streptomyces* sp. AC 3, *Streptomyces* sp. AC 20, *Streptomyces* sp. AB 11 (actinobacteria), *Candida butyri* 8(4), *Rhodotorula mucillaginosa* 4(1), *Sporopachydermia lactativora* 2(3) (yeasts), and *Arthrobotrys oligospora* DSMZ 2023, *Chaetomium globosum* Ch 10, *Phanerochaete chrysosporium* ATCC 42538, *Trichoderma atroviride* T14 (microfungi).

The microbial strains used in this study were isolated from wet husks (originated from the oil extraction process of olives cv, Leccino, Moraiolo, and Frantoio), except for *A. oligospora* DSMZ 2023, obtained from German Collection of Microorganisms and Cell Cultures, and *P. chrysosporium* ATCC 42538, obtained from American Type Culture Collection.

The use of microbial starters for the biotransformation of wet husk was the one described by Echeverria et al. (2012) for both industrial pilot and farm level. At the industrial pilot level, the composting was run for 90 days with 1.2t of wet husk, maintaining the humidity of the piles (200 kg each) without starters (A, B, C) and with starters (D, E, F) at 40–60%. During the composting period, the piles were mechanically turned every time the internal temperature reached or exceeded 55–60 °C to allow aeration and to decrease temperature.

At farm level the composting process was carried out by Cooperativa Arnasco in Arnasco (Savona, Italy) in an olive oil mill that serves a 25–30 year-old 40 ha olive grove (cultivars Taggiasca and Pignola). Twenty tons of wet husk from a two-phase decanter extraction process were mixed with the starters, added with 1 ton of olive wood chips to maintain aereation and placed in a windrow pile. The mechanical turning of the pile was done occasionally, depending on the external temperature. Sampling was carried out in compliance with the methods of the Italian Ministry of Agriculture (Legislative Decree 75/2010) at time 0, 15, 60 and 90 days for composting at industrial pilot, and at time 0, 35, 100, 145 and 200 days for composting at farm level. The temperature profile of the piles and the physical–chemical traits of the samples from the industrial pilot composting process were reported in a previous paper (Echeverria et al., 2012). The methods to determine the physical–chemical traits of samples from the farm scale composting process, to extract the humic substances and to express the humification index (HI) are those described by Echeverria et al. (2011).

2.2. Microbial cultures, DNA extraction and PCR amplification

DGGE markers were prepared with the same microbial strains as the starters with the addition of *Streptomyces* sp. ABT42, *S. lactativora* 2(3), *C. globosum* ChT3. Bacteria and actinobacteria were maintained on Nutrient agar (Oxoid, Milan, Italy), yeasts and fungi on Malt agar (Oxoid, Milan, Italy).

Genomic DNA was extracted from bacteria, actinobacteria and yeasts liquid cultures grown overnight at 25 °C using "MasterPure™ Yeast DNA Purification" (Epicentre) according to the manufacturer's protocols. For the DNA extraction from fungi, the mycelium grown on plates was resuspended with 10 mL deionized water. 2 mL of suspension were inoculated in 40 mL of Malt Broth for an overnight incubation on a rotatory shaker at 25 °C. After harvest by centrifugation at 10,000 rpm and 3 washes with $MgCl_2$ 0.1 M, 400 mg of mycelium (f.w.) were transferred into a 2 mL eppendorf tube and crushed. The other steps were the same as for bacteria and yeasts.

DNA extraction from compost samples was carried out using the "PowerSoil™ DNA Isolation Kit" (Mo-Bio Laboratories San Diego, CA) according to manufacturer's instructions. For the analysis of the bacterial community, the amplification of the variable region V3–V5 of 16S rDNA was carried out using the primers 341F (CCTACGGGAGGCAGCAG) and 907R (CCGTCAATTCCTTTRAGTTT) (Yu and Morrison, 2004). The primer 341F had at its 5′ end an additional 40-nucleotide GC-rich tail (5′-CGCCCGC CGCGCCCC GCGCCCGTCCCG CCGCCCCCGCCCG-3′). Amplification reaction was prepared in a final volume of 50 μL, using 1 μL of extracted DNA diluted 1:100, 10 μL of 5× Phusion™ HF Buffer (Finnzymes), 1 U of Phusion™ High-Fidelity DNA polymerase (Finnzymes), 0.2 mM of each dNTPS (GeneAmp dNTP Mix, Applied Biosystem), 0.5 μM of each primers (Primm) and 3% DMSO (Finnzymes). The fragment obtained is 560 bp long. The reaction was carried out using an iCycler-iQ Multicolor Real-Time PCR Detection System (Biorad) with the following denaturation, amplification and extension procedure: 98 °C 30 s; 98 °C 10 s, 52 °C 10 s, 72 °C 15 s for 35 cycles; 72 °C 10 min. The presence of amplicons was confirmed by electrophoresis in 1.5% (w/v) Agarose I (Euroclone) in TBE 1× buffer (Euroclone) gels stained with ethidium bromide 0.5 μg mL^{-1}. All gels were visualized and captured as TIFF format file by Liscap program for Image Master VDS system (Pharmacia Biotech). For the analysis of fungal community, the amplification of the variable region D1–D2 of 26S rDNA was carried out using the primers NL1 (GCATATCAATAAGCGGAGGAAAAG) and LS2 (ATTCCCAAACAACTCGACTC) (Cocolin et al., 2000). The primer NL1 had at its 5′ end an additional 40-nucleotide GC-rich tail. Amplification reaction was prepared in a final volume of 50 μL, using 2 μL of extracted DNA diluted 1:10, 10 μL of 5× Phusion™ HF Buffer (Finnzymes), 1 U of Phusion™ High-Fidelity DNA polymerase (Finnzymes), 0.2 mM of each dNTPS (GeneAmp dNTP Mix, Applied Biosystem), 0.5 μM of each primers (Primm) and 3% DMSO (Finnzymes). The fragment obtained is 250 bp long. The reaction was carried out using an iCycler-iQ Multicolor Real-Time PCR Detection System (Biorad) with the following denaturation, amplification and extension procedure: 98 °C 30 s; 98 °C 10 s, 48 °C 10 s, 72 °C 15 s for 35 cycles; 72 °C 10 min. The presence of amplicons was confirmed with the same procedure as for bacterial communities.

2.3. DGGE analysis of bacterial and fungal community

The amplicons were analyzed using the DCode™ Universal Mutation Detection System BIORAD. 20 μL of the PCR products