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Enzymatic characterization of microbial isolates from lignocellulose waste composting: Chronological evolution

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ABSTRACT

Successful composting is dependent upon microbial performance. An interdependent relationship is established between environmental and nutritional properties that rule the process and characteristics of the dominant microbial communities. To reach a better understanding of this relationship, the dynamics of major metabolic activities associated with cultivable isolates according to composting phases were evaluated. Ammonification (72.04%), amylolysis (35.65%), hemicellulolyis (30.75%), and proteolysis (33.61%) were the more frequent activities among isolates, with mesophilic bacteria and fungi as the prevalent microbial communities. Bacteria were mainly responsible for starch hydrolysis, while a higher percentage of hemicellulolytic and proteolytic isolates were ascribable to fungi. Composting seems to exert a functional selective effect on microbial communities by promoting the presence of specific metabolically dominant groups at each stage of the process. Moreover, the application of conglomerate analysis led to the statement of a clear correlation between the chronology of the process and characteristics of the associated microbiota. According to metabolic capabilities of the isolates and their density, three clear clusters were obtained corresponding to the start of the process, including the first thermophilic peak, the rest of the bio-oxidative stage, and the maturation phase.

1. Introduction

Composting is defined as a biotransformation process in which solid organic matter turns into mature and stabilized material by means of microbial action in aerobic conditions. The final product, compost, is a noteworthy soil amendment (Ros et al., 2006), a valuable substrate for soilless culture (Martínez et al., 2013), and an efficient tool for bioremediation processes (d'Errico et al., 2013) or soil restoration (Tejada et al., 2009b). Such applications are related to compost capability for improving soil's physical, chemical and biological properties (Ozores-Hampton et al., 2011), which in turn results in higher soil fertility and productivity.

Microorganisms play a key role both in the composting process and in compost properties. On the one hand, most of the transformations that organic matter undergo, particularly during the bio-oxidative stage, are ascribable to microbial action (Federici et al., 2011), and, on the other hand, effects derived from compost incorporation into soil strongly depend upon microbial populations in the final product (Farrell et al., 2010). It can be said, therefore, that proper performance of the process and quality of the final product lie on qualitative and quantitative composition of microbial communities associated to different stages (Pepe et al., 2013).

Several reports concerning dynamics and characterization of composting microbiota have been published throughout the years. Nevertheless, available information is not as thorough as would be desirable. On the one hand, diversity regarding composting substrate is limited, since most reports are focused on the valorization of municipal solid wastes and, to a lesser extent, in biosolids (Bonito et al., 2010; Khalil et al., 2011). Taking into account the influence that nature and properties of substrate have cause on microbial populations in composting (Ishii and Takii, 2003), processes with different starting materials represent unique environments from a microbiological point of view. Thus, a universal microbial pattern representative of every condition is not available. Knowledge of a specific process demands individual analysis.

On the other hand, there is a paucity of literature describing the complete biochemical characterization of the microbiota involved in the process. Some reports monitor the enzymatic evolution of the process (Tejada et al., 2009a), and others identify specific microbial communities (Li et al., 2013b). A reduced number of studies

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set up a connection among various profiles (Wei et al., 2012). Finally, some studies characterize one or a limited group of isolates (Eida et al., 2012). To our knowledge, no papers monitoring the enzyme dynamics of the process according to biochemical characterization of isolates exist.

Relevant metabolic capabilities of microorganisms in relation to organic-waste transformation are diverse. On the basis of the specific nature of the residual material, particular enzymatic features will be more significant in the biodegradation reactions. Nevertheless, general enzymatic systems involved in the transformation of main polymeric macromolecules always take part in the process. Thus, metabolic activities such as protein degradation, lipid modification, or even lignocellulose transformation are associated with the composting process in relation to whatever the properties of the starting material are, since they are of universal distribution. By contrast, other activities depend upon the properties of the starting material (Vaz-Moreira et al., 2008). In a plant-waste-based composting processes, activities such as pectin degradation or starch hydrolysis potentially gain importance on account of residue composition, becoming as predominant as more common enzymes (Ajila et al., 2012). The characterization of the dynamics of microbial functionality during the different stages of composting not only would provide a better understanding of the evolution of the process from a microbiological perspective and a more complete overview of connections between biotic and abiotic factors, but would also provide a tool for mining microorganisms useful in different biotechnological fields (Amore et al., 2013; Vargas García et al., 2012).

The aim of this work was to study the dynamics of culturable microbiota according to its metabolic capability profile during composting of lignocellulosic waste. A detailed analysis of metabolic potential of microorganisms throughout the different stages in the biotransformation process could contribute to gaining knowledge and understanding of the microbial role in composting and the influence of process conditions in the composition of the microbial population.

2. Material and methods

2.1. Composting procedure and sampling

Sun-dried tomato plants and pinewood chips, ground to $\leq$3 cm size, were mixed in 1:1 (w/w) proportion (C/N ratio: 25) and disposed as a trapezoidal pile on a cement platform equipped with a basal-forced aeration system. The pile was built up using about 500 kg of starting material and distributed according to following dimensions: 1.5 m width $\times$ 3 m length $\times$ 1 m height. Throughout the process, temperature was monitored using a long-handled (50 cm) thermometer probe PT100 model MPT2 (Lexitron-Guemisa, Madrid, Spain), while moisture content and aeration status were kept at prearranged levels, between 45 and 50% and over 10% oxygen, respectively, during the bio-oxidative stage. Moisture content was corrected by watering when necessary, and an air supply was provided through the basal system (perforated PVC tubes connected to a pump Lowarda CEAM-7013, Montecchio Maggiore, Italy) with a regime of aeration of 0.6 m s^{-1} every 4 h for 5 min. Additional mechanical-turning operations were carried out on the basis of the thermal profile to promote the mixing of the material and reactive microbial activity.

A thorough sampling strategy was programmed in order to achieve the most accurate scenario. According to thermal values, 19 samples were collected at different times of the process (Table 1). At every sampling time, nine locations were set and distributed along the three spatial dimensions (width: 25, 75, and 125 cm; length: 50, 150, and 250 cm; height: 10, 50, and 90 cm). Subsamples were equally mixed to a final weight of 1 kg and split into three replicates. Replicates were fractionated and treated according to specific protocols: air-dried at 40 °C overnight, ground to <1 mm for chemical analyses, and freshly processed for microbial isolation, moisture, and pH determination.

2.2. Chemical and physical analyses

The moisture content was determined by drying at 105 °C for 24 h. The pH was analyzed in a 1:10 (w/v) water extract. Total carbon (C) and nitrogen (N) were determined in solid samples by dry combustion at 950 °C using a LecoTruSpec C–N Elemental Analyzer (Leco Co., St. Joseph, MI, USA). Organic-matter content was assessed by determination of loss on ignition at 550 °C to a constant weight.

2.3. Microbial analysis

Mesophilic and terrmophilic microbiota (bacteria, actinobacteria, and fungi) were quantitatively estimated using a 10-fold serial dilution method. Initial suspension was obtained by adding 10 g of compost to 90 mL of sterile saline solution (NaCl, 0.9% w/v) and shaking for 30 min at room temperature. APHA and Rose Bengal Agar (Cultimed, Spain) were respectively used for bacteria and fungi, while actinobacteria was grown on Sodium Caseinate Agar (SCA) (composition in g L^{-1}: sodium caseinate 2.0; asparagine 0.1; sodium propionate 4.0; K_2HPO_4 0.5; $MgSO_4$ 0.1; $FeSO_4$ 0.001; glycerol 5.0; agar 15.0). Bacteria were incubated for 48 h and fungi and actinobacteria for 96 h, at 30 or 50 °C for mesophilic or thermotolerant microorganisms, respectively. Results were reported as colony-forming units (CFU) per gram of compost dry weight.

All different morphotypes identified in the plates were isolated in the conditions previously described for media and incubation. Isolates were regularly transferred to fresh agar slant and cryopreserved at −80 °C in cryoballs (Cryoinstant™, Deltalab, Barcelona, Spain) for long-term conservation.

2.4. Enzymatic analyses

All the isolates were investigated regarding their metabolic capability in relation to starch, pectin, lipid, and protein hydrolysis; lignocellulose fractions degradation; ammonification; and phosphate solubilization. Media used for the assay of each activity were as follows: starch agar for amilolytic activity (Vedder, 1915), Winogradsky medium with asparagine for ammonifying microorganisms (Pochon and Tardieux, 1962), Janshekar basal medium added to microcrystalline cellulose 0.5% and aniline blue-black 0.005% for cellulolytic activity (Janshekar et al., 1982; Kauri and Kushner, 1988), Janshekar basal medium added to xylan 0.5% for hemicellulolytic activity (Janshekar et al., 1982), basal medium added to poly R-478 dye 0.02% (Freitag and Morrell, 1992), basal medium added to tributyrin 1.0% (Leuschner et al., 1997), basal medium added to polygalacturonic acid 1% (Cotty et al., 1990), Janshekar basal medium added to sodium caseinate 1.0% for proteolytic activity (Janshekar et al., 1982) and NBRIP medium for phosphate-solubilizing activity (Nautiyal, 1999). All reagents were purchased from Sigma–Aldrich (St. Louis, Mo, USA). All assays were carried out on agar plates, except ammonification (MPN tubes). Every agar plate was used for testing eight different isolates. Bacteria and actinobacteria were assayed adding 25 μL droplets of microbial suspensions resulting from biomass grown on an agar slant for 48–72 h. In the case of fungi, pieces of 6 mm of diameter made with a hole puncher were disposed on each of the eight similar divisions in the plate. Tubes for ammonifying MPN were

inoculated using one mL of microbial suspension (bacteria and actinobacteria) and 6 mm diameter pieces (fungi).

All media were incubated at 30 °C for 48–72 h (amilolytic, proteolytic, and phosphate-solubilizing activities), 120 h (hemicellulolytic, lipolytic, and pectinolytic activities), 240 h (cellulolytic and lignolytic activities), and 400 h (ammonifying activity). Diagnosis reactions were based on the decolorization of the medium (cellulolytic and lipolytic activities), on the development of a clearance halo (hemicellulolytic, lipolytic, proteolitic, and phosphate-solubilizing activities) or a turbid halo (pectinolytic activity), the presence of a clearance halo after Lugol staining (amilolytic activity), and formation of an orange precipitate after the addition of Nessler reagent (ammonifying activity).

2.5. Data analysis

Results were qualitatively and quantitatively processed on the basis of composting stages. On the one hand, a number of positive isolates within samplings were estimated for every metabolic activity and expressed as the percentage of the total number of isolates. On the other hand, a quantitative percentage was also calculated taking into consideration the estimated density for positive isolates in relation to the total density.

Data were analyzed in order to know if they fit a normal distribution. If not, the Shapiro–Wilk test was performed on modified data to check suitability of the proposed transformation. Principal Component Analysis was used for reducing the number of variables according to the influence of their different lineal combinations. Cluster analysis was used to group data (Ward Method, Squared Euclidean) on the basis of similarity. All analyses were performed at a 95% confidence level using Statgraphics Centurion XVI version 16.1.17 (StatPoint Technologies, Inc., Warrenton, VA, USA).

3. Results and discussion

3.1. Composting process

The physico-chemical properties of the process evolved as expected in a typical composting transformation (Table 1). Thus, on the basis of the thermal profile, three typical stages were differentiated: bio-oxidative, cooling, and maturation phases. Within the bio-oxidative phase, three decreasing thermophilic peaks were observed, coinciding with the mechanical turnings. Thermal values in the following cooling stage were slightly higher than expected. Previous studies pointed out this different thermal profile during the cooling stage in the composting of lignocellullosic wastes (Vargas García et al., 2010), which might be a consequence of a residual microbial activity as a result of the remaining lignocellulose content of the composted materials (Wei et al., 2012) and its higher resistance to biodegradation (Hubbe et al., 2010). This activity can generate enough thermal energy to increase temperature over the ambient value, but not to the extent of producing a true thermophilic stage.

Values for pH ranged from 8.3 to 8.7, with the only exception being raw material (pH = 7). Similar results have been described for lignocellulosic wastes, whether they are co-composted with other types of residues (Albrecht et al., 2010a) or composted as the sole raw material (Echeverria et al., 2012). The maintenance of pH on values that are slightly alkaline supports the appropriate degradation rate (Milczarek et al., 2013).

C/N ratio and organic matter developed similar trends, showing mild but constant decreases during bio-oxidative and cooling stages and a marked drop in maturation. This pattern is in agreement with those described by Alburquerque et al. (2009) and Gigliotti et al. (2012), who attribute this evolution to the composition of the raw material and the high content of biodegradation-resistant fractions. Final values, 13.3 and 43.9 respectively, fit the requirements for stabilized lignocellulosic-based compost according to the Spanish regulations (RD 506/2013). Thus, on the basis of the analyzed physico-chemical parameters, the process progressed correctly. Consequently, the evolution of the microbial communities and their metabolic profiles can be considered representative of the composting of green lignocellulosic wastes.

3.2. Microbial isolates and metabolic activities

The isolation program allowed the production of a culture collection consisting of 1457 microorganisms. All of these were qualitatively checked regarding their metabolic profiles. Results are reported in Fig. 1. Ammonification was the most frequent activity, since up to 72% of the isolates were able to transform organic nitrogen into an ammonia or ammonium cation. This is the preferred nitrogen source for most microorganisms (Geisseler et al., 2010), and it shows the outstanding role of this community in habitats with high contents in organic nitrogen, such as compost piles. Thus, the presence of microbial populations mainly capable of

Table 1
Sampling chronology and physicochemical parameters evolution throughout the process.

Sample code	Sampling days	Phase	Temperature (°C)	Moisture (%)	pH	C/N ratio	Organic matter (%)
MPR	0	Raw materials	24.0	49.9 ± 1.2	7.0 ± 0.0	27.7 ± 2.4	65.9 ± 1.4
MESA1	1	1st Rising mesophilic	43.2	48.4 ± 1.1	8.4 ± 0.0	25.7 ± 1.2	62.9 ± 1.7
TER1A	2	1st Early thermophilic	64.9	51.1 ± 0.7	8.4 ± 0.1	24.8 ± 1.8	62.9 ± 3.3
TER1B	5	1st Late thermophilic	65.1	44.8 ± 0.7	8.3 ± 0.1	25.4 ± 2.2	58.5 ± 3.6
MESD1	7	1st Decreasing mesophilic	59.8	49.4 ± 1.4	8.6 ± 0.0	24.7 ± 0.7	58.3 ± 2.5
MESA2	8	2nd Rising mesophilic	40.4	49.9 ± 0.2	8.6 ± 0.0	24.5 ± 1.4	59.0 ± 3.2
TER2A	9	2nd Early thermophilic	59.4	45.7 ± 2.1	8.6 ± 0.0	24.5 ± 0.9	64.8 ± 0.5
TER2B	12	2nd Late themophilic	54.8	44.6 ± 0.8	8.7 ± 0.0	22.9 ± 1.9	61.9 ± 1.6
MESD2	14	2nd Decreasing mesophilic	45.9	41.0 ± 0.5	8.7 ± 0.0	23.5 ± 1.8	63.7 ± 2.1
MESA3	15	3rd Rising mesophilic	44.2	47.4 ± 0.5	8.7 ± 0.0	23.5 ± 1.7	62.7 ± 2.8
TER3A	16	3rd Early thermophilic	48.2	46.5 ± 0.8	8.7 ± 0.0	23.8 ± 1.3	62.5 ± 1.2
MESD3	26	3rd Decreasing mesophilic	31.3	48.7 ± 0.6	8.5 ± 0.1	22.7 ± 1.1	62.0 ± 0.3
MESA4	28	4th Rising mesophilic	34.8	47.4 ± 0.7	8.5 ± 0.1	20.6 ± 0.6	63.8 ± 4.6
MES5	42	Mesophilic (Cooling)	39.4	49.7 ± 0.3	8.7 ± 0.0	20.7 ± 1.9	59.5 ± 1.3
MES6	56	Mesophilic (Cooling)	40.0	41.0 ± 0.3	8.7 ± 0.0	21.6 ± 0.8	59.3 ± 2.6
MES7	63	Mesophilic (Cooling)	33.9	46.6 ± 2.5	8.6 ± 0.0	19.7 ± 0.8	59.2 ± 2.2
MAD1	119	Early maturation	38.5	50.9 ± 1.5	8.6 ± 0.0	17.3 ± 0.3	53.8 ± 1.6
MAD2	168	Late maturation	26.5	42.5 ± 1.7	8.5 ± 0.0	14.4 ± 0.5	48.0 ± 3.5
PRF	189	Final product	20.6	40.8 ± 1.8	8.5 ± 0.1	13.3 ± 0.7	43.9 ± 2.4

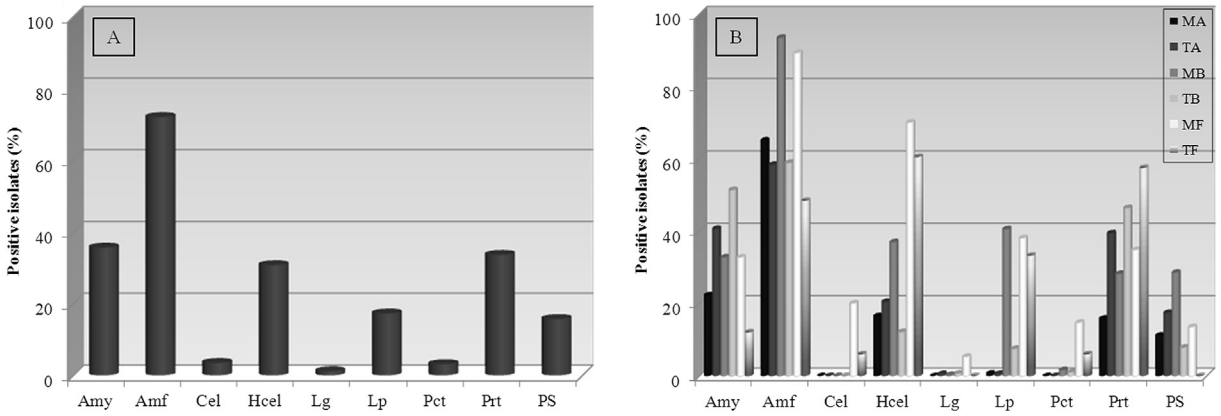

Fig. 1. Positive isolates, expressed as percentage, for the different metabolic activities tested. A: Absolute percentages; B: Microbial groups percentages; MA: Mesophilic actinobacteria; TA: Thermotolerant actinobacteria; MB: Mesophilic bacteria; TB: Thermotolerant bacteria; MF: Mesophilic fungi; TF: Thermotolerant fungi; Amy: Amylolysis; Amf: Ammonification; Cel: Cellulolysis; HCel: Hemicellulolysis; Lg: Lignolysis; Lp: Lipolysis; Pct: Pectin Hydrolysis; Prt: Proteolysis; Ps: Phosphate Solubilization.

mineralizing organic nitrogen is a usual feature of composting, as has been previously reported (Wang et al., 2011). Predominance of one or another microbial group (bacteria, actinobacteria, or fungi) within the ammonifying community depends on both physicochemical and nutritional properties of the specific habitat (Pepe et al., 2013). In this sense, C:N ratio is one of the most influential parameters. Bacteria demand higher N availability per unit C, which means they will be more intensively involved in the ammonification process at low C:N ratios (Högberg et al., 2007). Proteolytic activity is also associated to the N cycle and can be considered as the first step in the ammonification process (Körner and Stegmann, 2002). As previously described by Ryckeboer et al. (2003), thermotolerant proteolytic isolates were proportionally larger than the mesophilic ones. As some studies report (Chroni et al., 2009; Ishii et al., 2000), hydrolysis of proteins seems to prevail during the thermophilic phase, which might favor the existence of a larger proportion of proteolytic isolates among thermotolerant microorganisms.

Marked differences were detected among activities dealing with lignocellulosic and starchy polymer degradation. About a third of the total isolates showed both amilolytic and hemicellullolytic activity. By contrast, the percentage of microorganisms capable to hydrolyze cellulose, lignin, and pectin did not exceed 4%. It is generally accepted that starch and hemicellulose are easier hydrolysable polymers in contrast to cellulose or lignin (Pérez et al., 2002). Hemicellulose does not adopt a complex structure or present recalcitrant fractions in the same manner as cellulose and lignin, which limit enzyme activity or reduce the number of enzymes able to act on these polymers (Hall et al., 2010; Ruiz-Dueñas and Martínez, 2009). Moreover, lignocellulolytic microorganisms such as some cellulolytic bacteria do not always express these enzymatic systems (Schneider et al., 2012). With regard to the nature of the isolates, fungi were prevalent regarding activities associated to lignocellulose degradation. Although some prokaryotes are able to degrade lignocellulose, most of them take part in a secondary process associated with the transformation of oligomeric or monomeric compounds released from primary-degradation actions (Vikman et al., 2002). Thus, the most significant microorganisms involved in this process are fungi (Malherbe and Cloete, 2002), in contrast to amylase activity, which is widely distributed among both prokaryotic and eukaryotic microorganisms (Soni et al., 2003).

The proportion of lipolytic isolates was relatively low in comparison to other activities, although levels were slightly higher than those reported for similar environments (Cardenas et al., 2001). Distribution of this enzymatic activity among different microbial groups was unbalanced. Almost 40% of fungal and mesophilic bacterial isolates were positive for this activity, while lipolytic actinobacteria and termophilic fungi were hardly detected. As is the case for other enzymes, lipases are equally associated to prokaryotes and eukaryotes. Nevertheless, frequencies depend on specific characteristics of environment, and, therefore, different ratios among microbial groups can be observed on the basis of the origin of isolates (Ko et al., 2005). In recent years, phosphate-solubilizing microorganisms have attracted attention as tools for increasing the P-recovery efficiency in phosphate-bearing resources (Vassileva et al., 2010). Moreover, this feature is considered to be one of the main requirements for the so-called plant-growth-promoting microorganisms (Vassilev et al., 2006). Thus, the presence of this kind of microorganism in compost is of special interest because of its application as an organic amendment for the improvement of agricultural soils. A total of 231 isolates expressed P-solubilizing activity, which represented a proportion close to 16%. Most of the isolates were of prokaryotic origin, especially bacteria, while fungal isolates were minimal. This last result is attributable to the lack of thermotolerant P-solubilizing isolates, since the proportion of mesophilic fungi was in a similar range of other groups. Similar distribution was reported by Chang and Yang (2009) when analyzing the P-solubilizing microbial population associated with compost comprised of different raw materials. This prevalence of bacterial isolates may be a consequence of the source of calcium used for detection in the culture medium, tricalcium phosphate, since fungi seem to be more efficient on other types of calcium compounds (Oliveira et al., 2009).

3.3. *Tracking quantitative metabolic activities dynamic of the composting process*

Data regarding the metabolic profile of isolates were broken down according to composting stages. Fig. 2 shows results for the total number of positive isolates, as well as qualitative and quantitative percentages of assayed metabolic activities. As was expected, results were in accordance to global data previously described (Section 3.2). Thus, the limited presence of total cellulolytic, lignolytic, and pectin hydrolytic isolates caused no remarkable variations throughout the entire process. In contrast, other activities varied to a notable extent, especially regarding the quantitative percentage. Amylolytic, hemicellulolytic, lipolytic, and proteolytic activities quantitatively dominated as composting evolved, which points out a selective effect of the process on the

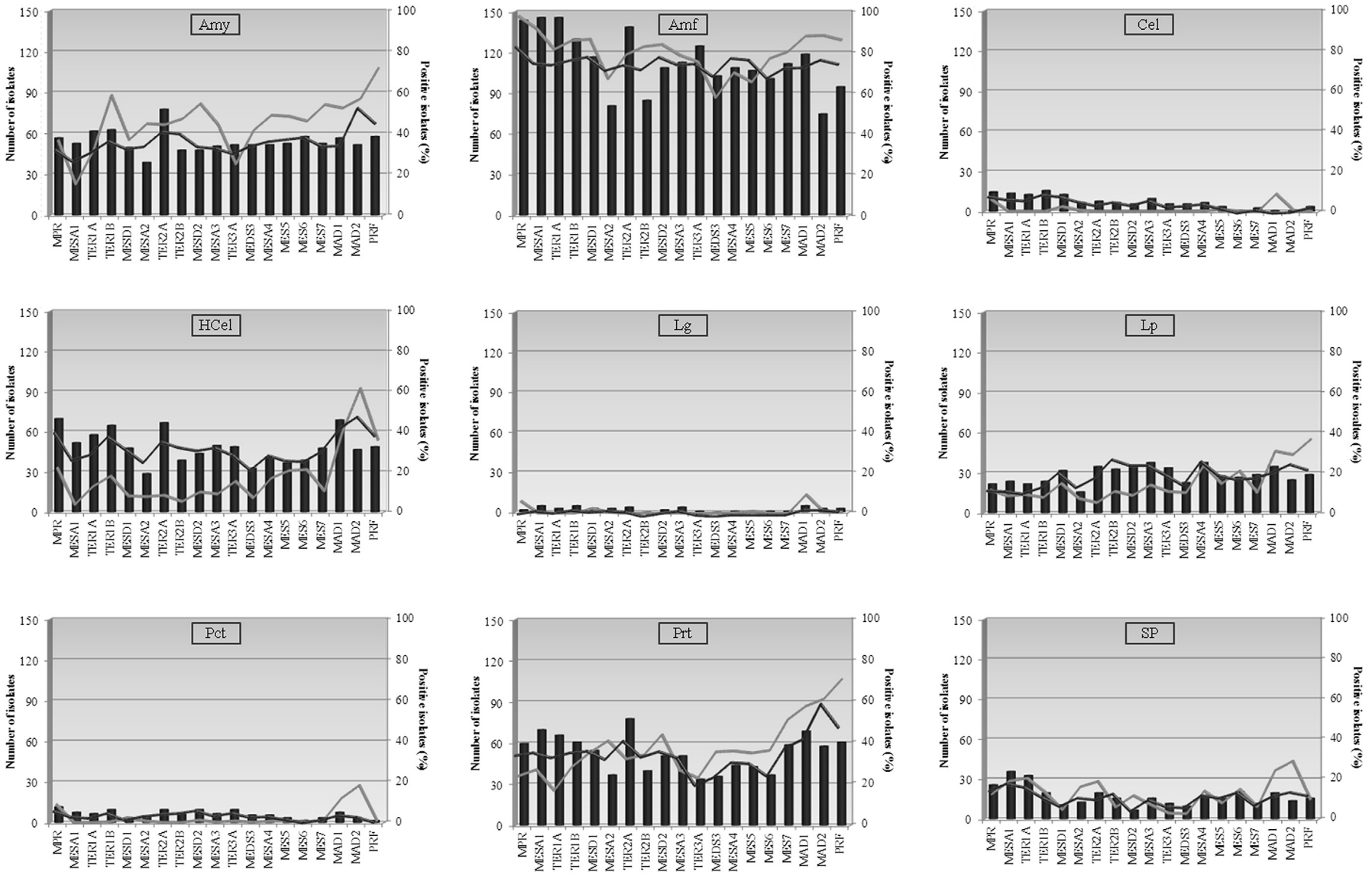

Fig. 2. Evolution of metabolic activities of isolates throughout the composting process. ▮ Bars: total number of isolates; Black line: qualitative percentage; Grey: quantitative percentage; Amy: Amylolysis; Amf: Ammonification; Cel: Cellulolysis; HCel: Hemicellulolysis; Lg: Lignolysis; Lp: Lipolysis; Pct: Pectin Hydrolysis; Prt: Proteolysis; Ps: Phosphate Solubilization.

microbial populations and their metabolic capabilities. This influence, well-known since composting has been studied, has been preferentially ascribed to temperature (Tang et al., 2007). However, this factor determines the presence of microorganisms mainly on the basis of their thermal tolerance, which obviously is not the case. Different parameters can affect microbial populations. Some of them, such as temperature, are common in all composting processes, regardless of what specific characteristics they have (raw-matter composition, pH, etc.). On the other side, there are properties conditioned by the specific nature of the material that also can affect the structure of the microbiota during composting (Federici et al., 2011). Hence, the response of microorganisms during the process might be split into two main components, one of them depending on materials and, therefore, difficult to generalize, and the other, more predictable, associated to the typical physico-chemical evolution of the process. In the process developed in this work, the characteristics of materials used (sun-dried tomato plants and pinewood chips) probably explain the evolution and composition of the microbiota and their associated biochemical activities to a higher extent than other common physico-chemical parameters. The study of Vaz-Moreira et al. (2008) supports this hypothesis, as it can be deduced by differences in the abundance of microbial communities on the basis of the raw material. Nevertheless, and regarding lignocellulose-degrading enzyme activities, increases seem to be usual as composting progresses in lignocellulose-based processes (Wei et al., 2012). A higher abundance of proteolytic microorganisms at the late stages of composting is also reported as a result of the accumulation of proteins released from death cells (Chroni et al., 2009). A similar trend was observed for amylolytic microorganisms, although peaks in the number and percentage of positive isolates were registered at the thermophilic stages, mainly for the two first phases (TER1 and TER2). Other authors have highlighted this correlation between high temperatures and increased amylolytic activity (Simujide et al., 2013).

The importance of the above-mentioned communities on the overall process is underlined by the Principal Component Analysis (PCA) applied to the results (Fig. 3). More than 55% of the variability was explained by two principal components (PC1 and PC2). In PC1, these communities showed maximal influence, hemicellulolytic parameters (number of isolates and microbial count percentages) being the most outstanding variables. In PC2, higher loads were ascribed to the ammonifying community, which was prevalent throughout the entire process. These microorganisms play a key role during the bio-oxidative stage, when organic nitrogen is converted into NH_3/NH_4^+, which is later immobilized, nitrified, or lost by volatilization (Li et al., 2013a). Nevertheless, they are present in high proportion in each and every one of the composting stages, even those in which NH_3/NH_4^+ levels are low (Pepe et al., 2013).

The increase in amylolytic, hemicellulolytic, lipolytic, and proteolytic communities was mostly ascribable to bacteria and fungi (Fig. 4), although thermotolerant actinobacteria contributed to some extent to amylolytic and proteolytic populations. Bacteria were prevalent among amylolytic microorganisms, while fungi accounted for the most lipolytic and, especially, hemicellulolytic communities. Neither bacteria nor fungi were prevalent regarding proteolytic activity, although fungi abundance was higher among mesophilic microorganisms, and bacteria were predominant among the thermotolerant ones. Thermotolerant bacteria were also in the majority regarding amilolytic activity. This prevalence of thermotolerant prokaryotic species within amylolytic and proteolytic species in composting was also reported by Ryckeboer et al. (2003).

In comparison to bacteria, a secondary role has been traditionally assigned to fungi in composting (Bonito et al., 2010), mainly because of the high thermal-value occurrence during the bio-oxidative stage. Nevertheless, this is open to question in some specific environments, as in lignocellulose-based processes (Insam et al., 2010). In such cases, the efficient enzymatic systems that fungi possess (Mohammad et al., 2012) favor a more prominent function for this group. On the other hand, their filamentous-hyphae growth provides fungi with a higher penetration capability and makes it easier to gain access to the complex lignocellulosic network (Gamauf et al., 2007). In contrast, bacteria have been reported to have a major role in starch hydrolysis in composting (Pascon et al., 2011), although fungi are also recognized as good amylase producers. Our results point to a similar conclusion, with bacteria representing the dominant amylolytic community. In particular, thermotolerant isolates showed the higher abundance in most of the samples, with actinobacteria also playing a leading role. Other studies reported a higher proportion of some enzyme capabilities, such as amylolysis and proteolysis, in thermotolerant

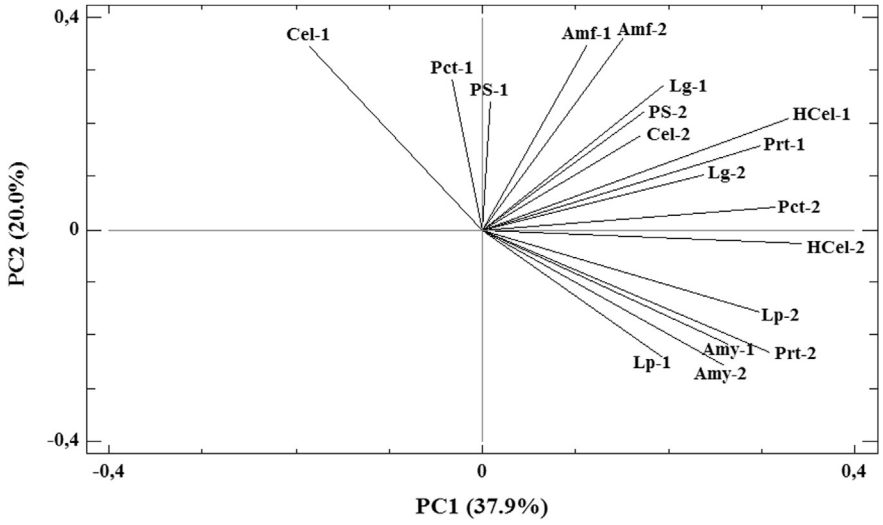

Fig. 3. Principal component analysis (PCA) of data showing percent load on the basis of two main principal components. Amy: Amylolysis; Amf: Ammonification; Cel: Cellulolysis; HCel: Hemicellulolysis; Lg: Lignolysis; Lp: Lipolysis; Pct: Pectin Hydrolysis; Prt: Proteolysis; Ps: Phosphate Solubilization; 1: Number of positive isolates percentage; 2: Positive microbial count percentage.

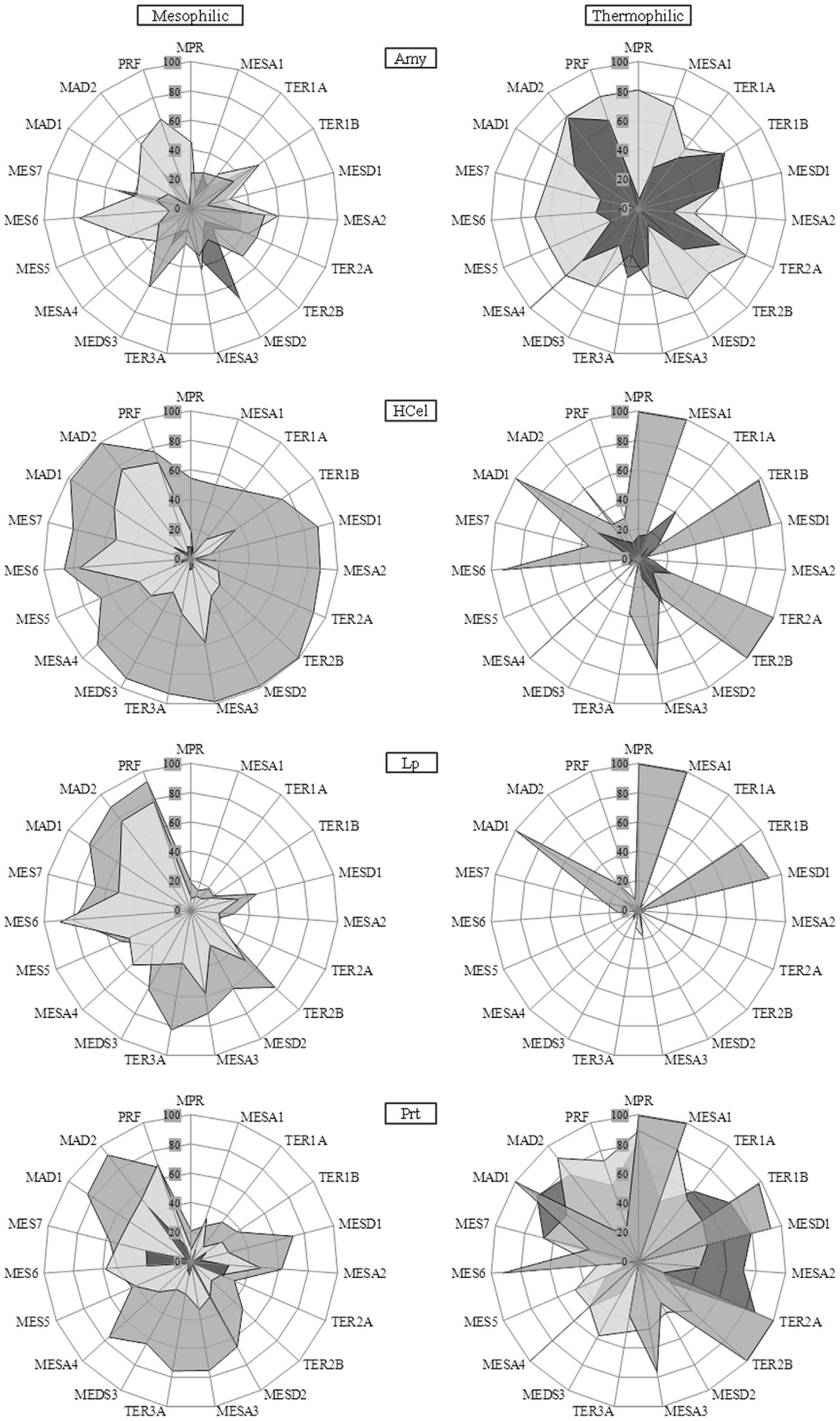

Fig. 4. Evolution of predominant metabolic activities within microbial groups. Results are expressed as quantitative percentage regarding total cell count. Amy: Amylolisis; HCel: Hemicellulolysis; Lp: Lipolysis; Prt: Proteolysis; ■: Actinobacteria; □: Bacteria; ▩:Fungi.

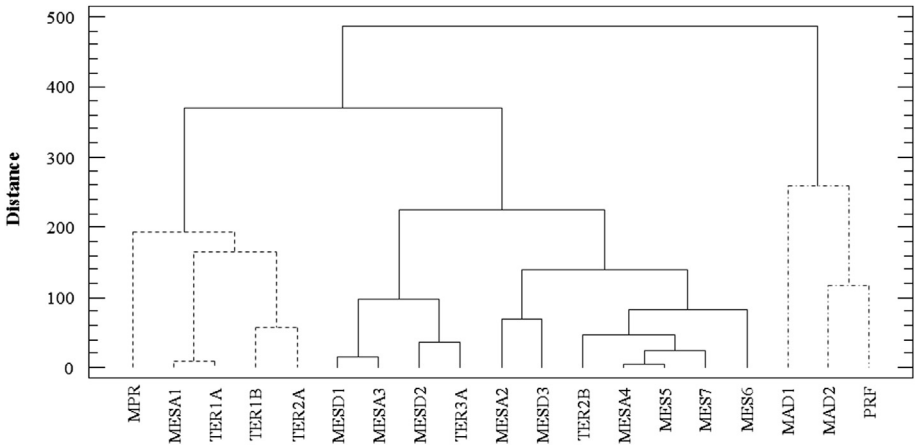

Fig. 5. Dendrogram showing groupings of composting phases according to distribution of qualitative and quantitative isolates metabolic capabilities on conglomerate analysis by the Ward Method using squared Euclidean as metric distance.

prokaryotic populations (Gorlach-Lira and Coutinho, 2007), which highlights the importance of starch-degrading enzymes in the metabolism of this type of microorganism (Bertoldo and Antranikian, 2002).

Bacteria were mostly responsible for the cumulative trend observed for some of the metabolic capabilities associated to the isolates, although fungi also contributed regarding proteolytic and lipolytic activities. Evolution of lipolytic isolates was especially remarkable, both in bacteria and fungi, since they were not detected in an important proportion until the second part of the bio-oxidative phase, after two weeks of composting. Gea et al. (2007) also observed a delay of the expression of lypolytic activity, which was probably ascribable to the initial predominance of processes associated to easily degradable compounds.

A conglomerate analysis of the qualitative and quantitative data regarding chronologic distribution of isolates according to their metabolic activities (Fig. 5) showed the existence of groupings in the composting process. Three main groups, clearly differentiated, were coincident with composting chronologic stages. Thus, the first group corresponded to the initial stage of the process, including the first thermophilic peak. The second group constituted samples belonging to the rest of the bio-oxidative phase as well as the cooling stage, and, finally, the third group included samples from maturation and the final product. This pattern clearly states the correlation between physico-chemical and nutritional properties prevalent in different stages and the functionally associated microbiota. Traditionally, this influence has been analyzed on the basis of individual parameters and the differentiation of their specific loads on the microbial evolution, including just the study of specific microbial communities or taking into consideration just phylogenetic data (Partanen et al., 2010; Zhang et al., 2011). By contrast, the number of papers reporting the existence of clearly differentiated groupings on functional microbial diversity is limited (Albrect et al., 2010b). All of these approaches are equally valid, since each and every one of them contributes to improving knowledge on composting. In this case, the existence of interdependence between composting evolution and chronological-microbial metabolic-activities distribution states a well-known but scarcely supported fact from a global point of view.

4. Conclusions

Composting cannot be understood without studying microbial communities and the role they play on the transformations taking place during the entire process. In the composting of lignocellulose-rich substrates developed in this work, prevalent activities among cultivable microorganisms were ammonification and hydrolysis of starch, hemicellulose, and proteins, with a dominance of mesophilic bacteria and fungi as the most active isolates. The low temporal variability of the results on cellulolytic, lipolytic, and pectinolytic activities, as well as the low levels in any activity observed in the case of actinobacterial isolates, should be further investigated. More in-depth studies would be needed to confirm or refute these findings.

On the other side, a quantitative cumulative effect in the most outstanding activities was observed throughout composting, resulting in the presence of a higher density of microorganisms exhibiting these activities at the final stage of the process and pointing out the existence of a functional selectivity of the process.

Although these results cannot be extrapolated, since conditions are unique for each process, general trends can be assumed. Thus, data described herein confirm the overall correlation between composting chronology and temporal distribution of microbial populations, according to their metabolic capabilities.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jenvman.2014.06.019.

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