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Exploiting composting biodiversity: Study of the persistent and biotechnologically relevant microorganisms from lignocellulose-based composting

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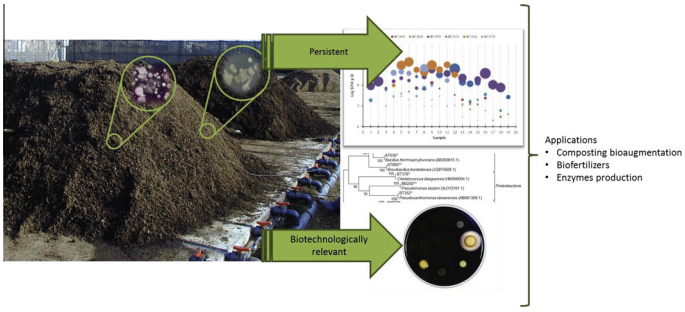
HIGHLIGHTS

- The microbial community of lignocellulose-based composting was evaluated.
- Microorganisms that persist throughout composting were identified.
- Microorganisms from composting of biotechnological interest were selected.
- The capability of selected isolates for composting bioaugmentation was studied.

GRAPHICAL ABSTRACT



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ABSTRACT

The composting ecosystem is a suitable source for the discovery of novel microorganisms and secondary metabolites. This work analyzes the identity of microbial community that persists throughout lignocellulose-based composting, evaluates their metabolic activities and studies the capability of selected isolates for composting bioaugmentation. Bacterial species of the phyla *Firmicutes*, *Actinobacteria* and *Proteobacteria* and fungi of the phylum *Ascomycota* were ubiquitous throughout the composting. The species *Arthrobacter russicus*, *Microbacterium gubbeenense*, *Ochrocladosporium frigidarii* and *Cladosporium lignicola* are detected for the first time in this ecosystem. In addition, several bacterial and fungal isolates exhibited a wide range of metabolic capabilities such as polymers (lignocellulose, protein, lipids, pectin and starch) breakdown and phosphate-solubilization that may find many biotechnological applications. In particular, *Streptomyces albus* BM292, *Gibellulopsis nigrescens* FM1397 and FM1411, *Bacillus licheniformis* BT575, *Bacillus smithii* AT907 and *Alternaria tenuissima* FM1385 exhibited a great potential as inoculants for composting bioaugmentation.

1. Introduction

Composting is a self-heating, aerobic process of bioconversion of organic waste into humus-like substances called compost. It constitutes one of the most sustainable methods for the management of solid organic waste in agroecosystems. The process involves the biotransformation of the organic matter by a complex microbial community whose structure changes depending on type and amount of nutrients, temperature, pH, aeration and water content (Hiraishi et al., 2003; Anastasi et al., 2005; Partanen et al., 2010; Federici et al., 2011). In a typical

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composting process the aerobic microbial metabolism drives temperature increase above 50 °C, followed by high temperatures that are maintained with fluctuations until most biodegradable nutrients are depleted. Then, the material gradually cools down because the microbial activity slows down and the organic matter stabilizes (López-González et al., 2013). The resident microbial community includes mesophilic and thermophilic bacteria and fungi that continuously adapt to the changing nutrient supply and altering environmental conditions. The effectiveness and speed of the process largely depends on the metabolic activities of microorganisms. Thus, the knowledge of the microbial composition and structure of the composting ecosystem is needed to control the process and to determine the quality and field of application of compost, and this has to be done for each specific process. Although some microorganisms have a widespread presence in composts, others may be more or less abundant according to the nature of raw materials and composting conditions (Anastasi et al., 2005; Partanen et al., 2010).

The recent application of molecular biological techniques to investigate the diversity of compost communities has revealed sequences of previously unsequenced and possibly uncultured 'new' microorganisms (Partanen et al., 2010; de Gannes et al., 2013). At the same time, data demonstrating the dominant occurrence of culturable microorganisms from compost have been reported (Dees and Ghiorse, 2001; Anastasi et al., 2005; Chandna et al., 2013). Although molecular techniques are of increasing use their application to complex environmental matrices such as compost still present some constraints (Bent and Forney, 2008; Chandna et al., 2013). Thus, isolation of microorganisms remains an essential task both to relate taxonomic and metabolic diversity of organisms and to recover relevant species for further use. In fact, composting ecosystem has been considered a rich source for the isolation of microorganisms that are useful as e.g. inoculants for composting (Vargas-García et al., 2006) and producers of enzymes that hydrolyze polymers or degrade recalcitrant compounds (Federici et al., 2011). Despite the broad research in this field, the potential of this ecosystem for the discovery of novel microorganisms and secondary metabolites is far from being fully exploited, much more considering that each process and raw materials may provide different strains.

Microorganisms able to degrade polymers through the production of extracellular enzymes may use a wide range of carbon sources during the composting process, and thus they have a nutritional advantage. In lignocellulose-based composting, the enzymes they secrete to breakdown lignocellulose may also be useful in industrial processes such as bleaching in the pulp and paper industry, bioremediation of polluted soils, clean-up of wastewater effluents, and the production of second and third generation biofuels (Turner et al., 2007). One of the primary advantages of using these species for enzyme production lies in their capability to adapt to changes in the growing conditions. Thermophilic microorganisms are of special interest since they are potential sources of thermostable enzymes that have many advantages over most common products from mesophilic microorganisms (Turner et al., 2007; Olson et al., 2012).

This paper describes an attempt to identify and select specific microorganisms involved in the composting of lignocellulosic materials and exploit the isolated microorganisms for diverse uses such as enzyme production, compost inoculants and composting bioaugmentation. The overall aims of this study were (i) to analyze the microbial community that persists throughout composting, (ii) to evaluate the metabolic activities of biotechnological interest of the microorganisms from compost and (iii) to study the capability of selected isolates for composting bioaugmentation.

2. Methods

2.1. Composting and sampling

Composting of lignocellulosic materials (sun-dried tomato plant waste and pine woodchips at a 1:1 (w/w) ratio) and sampling was performed as indicated by López-González et al. (2013). Briefly, the mixture was composted in trapezoidal piles (1.5 m width × 3 m length × 1 m height) of about 500 kg for 42 days with forced aeration (bio-oxidative stage), and then allowed to cool and cure for additionally 147 days. The heap was turned three times during the process.

Approximately 1 kg of homogeneous composite sample from nine sampling sites was collected from the windrow at 19 different times, corresponding to distinct stages of composting (Table 1). Each sample was a homogeneous replicate and a representative mixture of the heterogeneity of the heap. All samples were freshly processed for microbial counts and isolation.

2.2. Counts, isolation and maintenance of microorganisms

Mesophilic and thermophilic bacteria, actinobacteria and fungi were estimated as described by López-González et al. (2013). Ten grams of compost were suspended in 90 mL of sterile saline solution (0.9% w/v NaCl) and shaken for 30 min at room temperature. Ten-fold serial dilutions were made in sterile saline solution (0.9% w/v NaCl) and 100 μL of appropriate diluted suspension was spread out in specific medium for each microbial group: Plate Count agar APHA (Cultimed, Spain) for bacteria; Rose Bengal Chloramphenicol agar-RB (Cultimed, Spain) for fungi; and Actinomycete Isolation Agar (AIA) (Difco, USA) for actinobacteria. The plates were incubated at 30 °C (mesophiles), and 50 °C (thermophiles) for 48 h (bacteria) or 96 h (fungi and actinobacteria). Based on size, morphology and pigmentation, different colonies were counted. Total group and morphotype counts were expressed as logarithm of colony forming units per gram of sample dry weight (Log CFU/g dw). Pure cultures of colony morphotypes were obtained on PDA (fungi) or APHA (bacteria and actinobacteria) plates (Supplementary material, Fig. S1).

Isolates were tentatively grouped and dereplicated by observing their morphological characteristics on plates. For comparison

Table 1
Samples collected during composting of lignocellulosic waste (López-González et al., 2013).[a]

Sample code	Composting days	Stage description	Stage code	Temperature (°C)
1	0	Raw material	RM	24
2	1	Mesophile	MES	43
3	2	Thermophile	THER	65
4	5	Thermophile	THER	65
5	**7**	**Thermophile**	**THER**	**60**
6	8	Mesophile	MES	40
7	9	Thermophile	THER	59
8	12	Thermophile	THER	55
9	**14**	**Thermophile**	**THER**	**46**
10	15	Mesophile	MES	44
11	16	Thermophile	THER	48
12	**26**	**Cooling**	**COO**	**31**
13	28	Cooling	COO	35
14	42	Cooling	COO	38
15	56	Cooling	COO	40
16	63	Cooling	COO	34
17	119	Maturation	MAT	39
18	168	Maturation	MAT	27
19	189	Final product	FP	21

[a] Turning of piles was performed after collection of samples marked in bold.

between colony morphotypes and counting, the plates were photographed with a Canon EOS 450D camera, with lens Zoom EF-S18-55 mm f/3.5–5.6 IS. Many colonies that were similar in color, shape, and size were observed with bright field microscopes, which allowed them to be segregated into distinct isolates. Bacterial presumptive differentiation was carried additionally by the use of Gram-staining, oxidase and catalase tests. All isolates were preserved on slants at 4 °C and cryoballs Cryoinstant (Deltalab, Spain) at −80 °C.

2.3. Analysis of activities of biotechnological relevance

The isolated microorganisms were tested for the expression of nine activities of biotechnological interest: ligninolytic, cellulolytic, hemicellulolytic, amylolytic, pectinolytic, proteolytic, lipolytic, ammonifying and phosphate-solubilizing activities (Supplementary material, Fig. S1). Ammonifying activity test was performed in liquid medium while the other assays were performed on plates with solid medium containing the appropriate substrate for the specific activity as described below. For bacteria and actinobacteria the plates were inoculated with 25 μL droplets of biomass suspension made in 500 μL sterile saline solution (NaCl 0.9%) from microorganism grown on one APHA agar slant for 48–72 h. The liquid medium (Ammonifying activity test) was inoculated with 1 mL of the suspension. In the case of fungi, pieces of 6 mm diameter from a 96 h culture on PDA were placed in the plates or the liquid medium. The inoculated media were incubated at 30 °C (mesophiles) or 50 °C (thermophiles) for 3 days (amylolytic, proteolytic and phosphatase-solubilizing activities), 5 days (pectinolytic, hemicellulolytic and lipolytic activities), 7–10 days (cellulolytic and ligninolytic activities) and 15 days (ammonifying activity) and checked for the presence of the activity. No discrepant results were recorded in repeated experiments.

Cellulolytic and ligninolytic microorganisms showed decolorization around the colony grown on 0.5% cellulose plus 0.005% aniline blue black- (Kauri and Kushner, 1988) and Poly R-478-containing (Freitag and Morrell, 1992) agar plates, respectively. A clear halo around colonies demonstrated hemicellulolytic activity on 0.5% xylan-containing medium. Amylolytic microorganisms grown on medium containing 1% soluble starch exhibited a clear halo around the colony on a purple background after the plates were flooded with 1 mL of iodine solution (Hankin and Anagnostakis, 1975). Lipolytic activity was demonstrated by a clear halo around the colony on medium containing 1% tributyrin (Leuschner et al., 1997). Proteolytic activity was indicated by the presence of a clear halo around the colony grown on medium supplemented with 1% sodium caseinate (Strauss et al., 2001). Pectinolytic microorganisms exhibited a clear halo around the colony grown on medium containing 1% polygalacturonic acid after the plates were flooded with 1 N HCl (Cotty et al., 1990). Phosphate-solubilizing activity was demonstrated in media containing 2.5% phosphate tricalcium by a clear halo around colony (Nautiyal, 1999). Ammonifying activity was analyzed in a liquid medium (9 mL) containing 0.02% asparagine according to Pochon and Tardieux (1962). The release of ammonium from the amino acid was detected by addition of Nessler reagent.

2.4. Biotransformation of organic matter

Isolates showing a suitable metabolic activity profile were tested for their capacity to biotransform organic matter at lab scale using solid substrate culture under non-sterile conditions. Two set of experiments were performed. The first was aimed at determining the most suitable inocula for organic matter breakdown that trigger the release of soluble monomers and/or the growth of microorganisms; and the second, by using the selected inocula, intended to mimic the mesophilic stage of a composting process, and the main chemical parameters that characterize the process were analyzed (Supplementary material, Fig. S1).

For the first set of experiments, lignocellulosic material (25 g of sun-dried tomato plant wastes ground to <3 cm) in a 1 L plastic container with a lid was inoculated with 3 mL of a suspension of the isolate biomass in saline solution (NaCl 0.9% w/v) and thoroughly mixed. The suspension was obtained by mixing biomass from three slants of the microorganism growing in APHA for 48 h (bacteria) or PDA for 96 h (fungi). The moisture of material was adjusted to 50% with water. Non inoculated controls were included as a reference. Three replicates were prepared for each treatment. The materials were incubated for 21 days at 30 °C. Samples were collected at the start of assay and after incubation for analysis of soluble organic carbon (SOC) and microbial biomass carbon (C_{mic}) as described below.

The assay was repeated in the same conditions in the second set of experiments by using selected inocula. Samples were also collected at the start and after 21 days for analysis of organic matter, total carbohydrates, reducing sugars, phenolic acids, lignocellulose fractions, and humic substances as described below.

The percentage of increase or loss (decrease) of component was calculated by considering the amount of component (per sample dry weight) at the start (Mi) and after 21 days incubation (Mf) as follows: % component loss = $((Mi - Mf)/Mi) \times 100$; % component increase = $((Mf - Mi)/Mi) \times 100$.

2.5. Analytical methods

Soluble organic carbon (SOC) was analyzed using a TOC–VCSN analyzer (Shimadzu Co., Kyoto, Japan) in an extract 1:4 (w:v) of sample in 0.5 M K_2SO_4 shaken at 200 rpm for 30 min and filtered through filter paper. Microbial biomass C (C_{mic}) was determined using the fumigation–extraction method according to Vance et al. (1987).

Organic matter content was assessed by determination of loss on ignition at 550 °C to a constant weight. For total carbohydrate analysis, samples (25 mg) were hydrolyzed with 0.1 mL of 12 M H_2SO_4 for 16 h at room temperature, followed by addition of 2.4 mL of distilled water and heating in boiling water for 8 h. Total sugars in the hydrolyzate were quantified by the procedure described by Dubois et al. (1956). Soluble reducing sugars were analyzed according to the method described by Somogyi (1952), in an extract obtained as indicated for SOC analysis.

Lignocellulose fractions (cellulose, hemicellulose and lignin) were analyzed by using a fiber analyzer Ankom (Ankom Technology, Macedon, NY, USA). Phenolic acids were quantified in pyrophosphate extracts from solid samples according to the method described by Marambe and Ando (1990). The humic fractions were extracted and determined according to the method described by Ciavatta et al. (1990), assessing humic-like carbon (HA) and fulvic-like carbon (FA) by analysis of total organic carbon in corresponding fraction in a TOC–VCSN (Shimadzu Co., Kyoto, Japan).

2.6. Identification of isolates

The identities of specific isolates were determined based on partial or nearly full length 16S rRNA gene (bacteria, including actinobacteria) and 5.8S-ITS region (yeast and fungi) sequence analysis. For bacterial genomic DNA extraction, freshly grown colonies were suspended in 500 μL of sterile milliQ water, heated at 97 °C for 5 min followed by exposure to ice bath for 5 min. The 1/10 diluted supernatant was used as the template. Fungi and yeast DNA was extracted by using plantDNAzol kit (Invitrogen) according to manufacturer guidelines. The amplification was carried out using MyCycler thermal cycler (Biorad). The 16S rRNA

genes of bacteria were amplified using universal primers: 27F (5′-AGAGTTTGATCATGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The 5.8S-ITS region of fungi and yeast was amplified using primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). The reaction mixture (20 μL) contained: 10 μL kit Mastermix 2,0X Taq DNA Polymerase 2.0 mM $MgCl_2$ (VWR), 0.06 μM each primer, and 2 μL of template DNA. The following thermal profile was used for the PCR: 95 °C for 2 min, 30 cycles (94 °C for 1 min, 55 °C for 1 min and 72 °C for 1.5 min), 72 °C for 10 min (for bacteria); 94 °C for 10 min, 30 cycles (94 °C for 1 min, 51 °C for 1 min, and 72 °C for 3 min), 72 °C for 10 min (for fungi). Amplified PCR products were checked by gel electrophoresis on 1% agarose gel in SB buffer (Invitrogen) supplemented with 0.002% GelRed (Biotium), and the bands were visualized under UV illumination in Gel Documentation system Gel DocTM XR (Biorad). Sizes of the amplicons were estimated in comparison with DNA ladder (50–10,000 bp Wide-Range DNA Marker, Sigma–Aldrich).The PCR products were purified using the Diffinity Rapid Tips (Sigma–Aldrich) and the clean DNA was sequenced by capillary sequencer ABI Hitachi 3500 Genetic Analyzer (Applied Biosystems). The forward and reverse sequences were edited, assembled and aligned using the programs Sequence Scanner v1.0 (Applied Biosystem), Reverse Complement (www.bioinformatics.org/sms/rev_comp.html), Clustal X v2.0.11, and MEGA 5 v5.2. The partial or nearly full length sequences were compared for similar nucleotide sequences with the BLAST search of the National Center of Biotechnology Information (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi). The strains closely related were retrieved from NCBI for further analysis.

For describing their phylogenetic relationship, the gene sequences were aligned by using Clustal X. A phylogenetic tree was constructed by means of neighbor-joining method using MEGA v 5. In order to estimate the confidence of the tree topologies, bootstrap resampling analysis for 1000 replicates was performed.

2.7. Statistical analysis

The data obtained in the study were the mean values of three replicates. A cluster analysis was used to group data (Ward Method, Squared Euclidean) out of treatments of lignocellulosic materials with inocula on basis of similarity. Principal component analysis (PCA) was used for data reduction of the chemical composting parameters obtained after treatment of lignocellulosic materials with inocula. All data analysis was performed using Statgraphics Centurion XVI version 16.1.17 (StatPoint, Inc., VA).

3. Results and discussion

3.1. Persistent microorganisms in composting

Microbial community was analyzed to reveal persistent mesophilic and thermophilic bacteria, actinobacteria and fungi during the composting. The criterion to select persistent microorganisms was its detection in eight or more samples out of 19 collected at different stages of the process (Table 1). Among the wide collection of isolates obtained (1457 isolates), only twenty five strains meet selection requirement. Counts of the persistent isolates and their relative abundance with respect to total group counts are shown in Fig. 1. According to the isolation medium (APHA-bacteria, RB-fungi or AIA-actinobacteria) and incubation conditions (30 °C-mesophilic or 50 °C-thermophilic), selected isolates included: two mesophilic (BM) and seven thermophilic (BT) bacteria; five mesophilic (AM) and two thermophilic (AT) actinobacteria; eight mesophilic (FM) and one thermophilic (FT) fungus. The two mesophilic bacteria (BM7 and BM24) were detected at levels ranging from 10^7 to 10^9 CFU g^{-1}, in 10 and 11 samples, respectively. Their relative abundance increased at cooling and maturation stages, reaching a maximum value of 11% of the total mesophilic bacterial community. Counts of selected thermophilic bacteria ranged between 10^6 and 10^9 CFU g^{-1} and they persisted for 8–18 sampling times, depending on the strain. Two of these isolates (BT309 and BT315) also dominated the thermophilic bacterial community in some stages, accounting for more than 40% of the total group count. The five selected mesophilic actinobacteria showed a decreasing trend both in total counts and relative abundance throughout composting. At early stages of the process some strains accounted for 20–30% of the total mesophilic actinobacteria community. Two thermophilic actinobacteria were detected in more than eight sampling times but their relative abundance never exceeded 13%. In contrast, several of the eight selected mesophilic fungi surpassed 25% of total fungal community at specific stages of the process. Also, the unique thermophilic fungus that meet sampling number criteria (FT1445), was clearly dominant at the early stage of the process, this isolate accounted for 40% of the total group community at day 1 of composting, but it never reached abundance percentages higher than 19% in further sampling times.

Table 2 summarizes the identification of the persistent bacterial and fungal taxa in compost based on sequence similarity, and their phylogenetic affiliation is shown in Fig. 2. The majority of the selected prokaryotic isolates (9 isolates) were affiliated with *Firmicutes*, followed by *Actinobacteria* (5 isolates) whereas only two isolates belonged to *Proteobacteria*. All fungi pertained to the phylum *Ascomycota*. Identification results revealed that some strains originally isolated and counted as actinobacteria (AM or AT) on AIA isolation medium were *Bacillus* instead. Actinomycete Isolation Agar (AIA) is selective for actinobacteria isolation because it is a relatively simple nutrient medium, but there are some reports that show other bacteria in addition to actinobacteria may also grow in this medium (Coombs and Franco, 2003).

The *Firmicutes* were represented primarily by *Bacillus* and related genera *Brevibacterium* and *Ureibacillus*, which were major bacterial community components at all stages (Fig. 1 and Table 2). *Bacillus* sp. are able to survive in the compost pile due to their capability to form endospores. Thus, it has been reported as a predominant genera present throughout the composting process (Dees and Ghiorse, 2001; Chandna et al., 2013), and the most dominant bacterial taxon recovered from compost feedstock (Ryckeboer et al., 2003). Three *Bacillus* species were detected, namely, *Bacillus licheniformis*, *B. subtilis* and *B. thermoamylovorans*. The first two species are commonly found in mesophilic and thermophilic stages (Ryckeboer et al., 2003; Federici et al., 2011), whereas *Bacillus thermoamylovorans* is detected especially during the thermophilic stage (Dees and Ghiorse, 2001). *Ureibacillus thermosphaericus* has also been previously isolated from compost (Vargas-García et al., 2006). In contrast, *Brevibacterium* is not a common culturable inhabitant of composting piles, but it has been detected as dominant taxon during mesophilic stage of lignocellulose-based composting by using culture-independent techniques (de Gannes et al., 2013).

The *Proteobacteria* have been usually reported to be minor microbial community constituents (Partanen et al., 2010; Chandna et al., 2013) but de Gannes et al. (2013) demonstrated this group has a notable prevalence in the mesophilic stage. In contrast, in the present work, two of the thermophilic bacteria selected (BT378 and BT352) belonged to this phylum. These were identified as *Chelatococcus daeguensis* (Alphaproteobacteria) and *Pseudoxanthomonas taiwanensis* (Gammaproteobacteria). Both isolates were detected in the intermediate stages of the process, including thermophilic, but not in the raw materials neither in samples collected at maturation and final product. Their relative abundance was

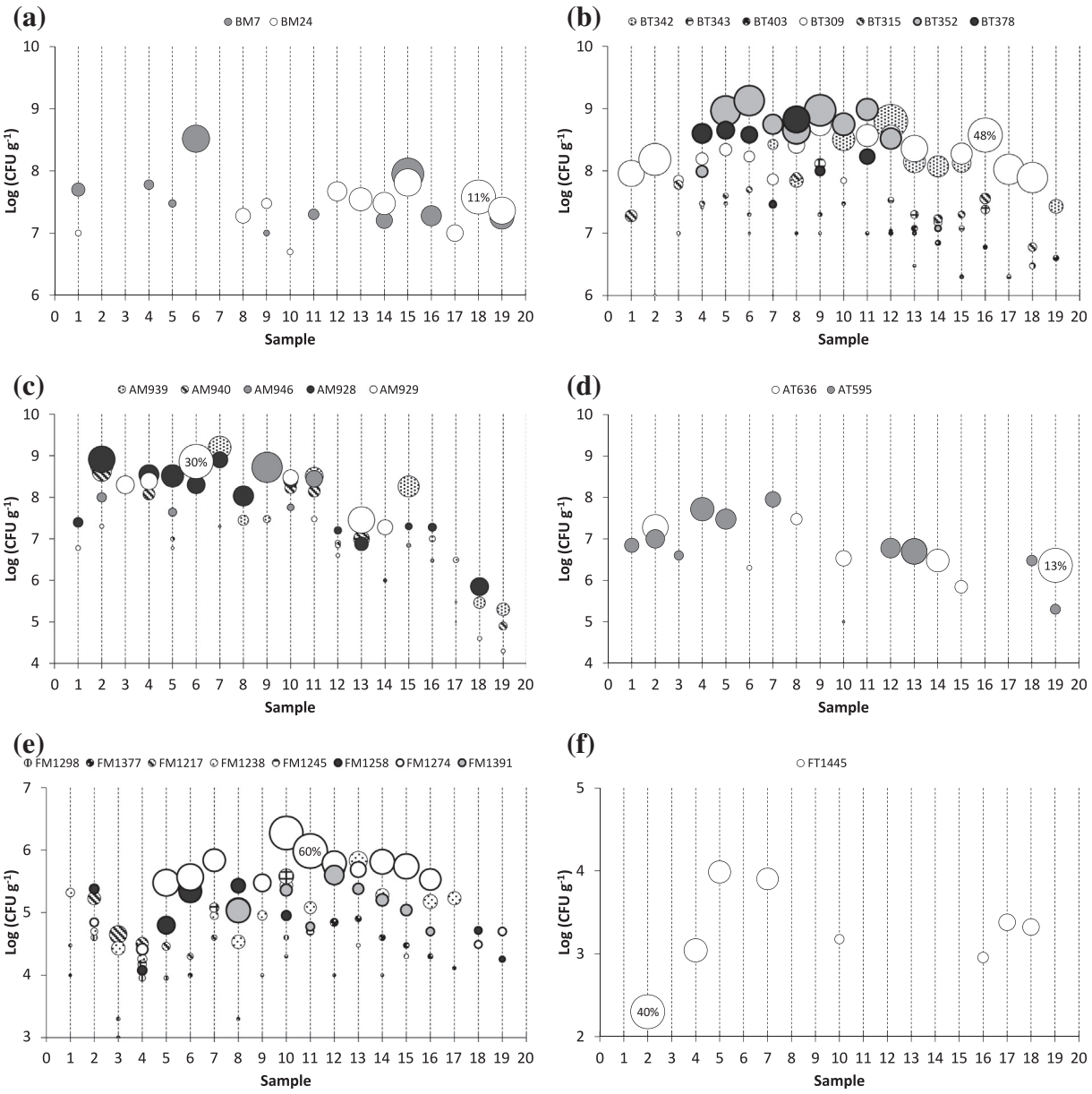

Fig. 1. Colony counts (Log CFU g^{-1}) and relative abundance [(isolate count/total group count) × 100] of the ubiquitous isolates throughout the composting process (see Table 1 for sample characteristics). Bubble sizes (diameter) represent relative abundance (%); maximum abundance bubble is included as a reference. Each graph shows data for isolates chosen within each microbial group analyzed: (a) BM, mesophilic bacteria; (b) BT, thermophilic bacteria, (c) AM, mesophilic actinobacteria; (d) AT, thermophilic actinobacteria; (e) FM, mesophilic fungi; (f) FT, thermophilic fungi.

quite high accounting for 29–40% of the total thermophilic bacterial community in sample 8 (collected at thermophilic phase after 12 days composting). Both genera have also been recently reported in lignocellulose-based composting (de Gannes et al., 2013).

Actinobacteria are typically abundant in compost, particularly at the thermophilic and curing stages (Ryckeboer et al., 2003; de Gannes et al., 2013). There were four *Actinobacteria* genera identified (*Microbacterium*, *Arthrobacter*, *Corynebacterium* and *Brachybacterium*), with the species *Microbacterium gubbeenense* and *Brachybacterium paraconglomeratum* as the most persistent. *B. paraconglomeratum* has been reported earlier in compost of household biowaste (Hiraishi et al., 2003). Although there are some reports on *Microbacterium* and *Corynebacterium* as inhabitants of composting pile (Ryckeboer et al., 2003; Federici et al., 2011), the species *M. gubbeenense* and *Corynebacterium casei* have not been earlier reported in this ecosystem. More surprising was the finding of the isolate BT403 that matched (99%) with *Arthrobacter russicus*. To the best of our knowledge, this is the first report of *A. russicus* culture in compost. This actinobacterium was isolated for the first time from the air and condensation water sampled in the Russian space station Mir. It grows well at 30 °C on BHI agar plates, but is unable to grow at 37 °C (Li et al., 2004). In the present study, BT403 was originally selected as a thermophilic bacteria growing at 50 °C. Although, it was not as abundant as other representatives of the thermophilic bacterial community (maximum relative abundance was 1.5% in sample 13 collected at cooling stage), it was detected in all composting stages except in maturation and raw materials. The persistence of this microorganism under harsh conditions of composting may relate to the capability of *Arthrobacter* to survive as vegetative cells during stress.

Fungi selected included species of the genera *Aspergillus*, *Cladosporium*, *Gibellulopsis*, *Ochrocladosporium*, *Plectosphaerella* and

Table 2
Persistent microorganisms isolated from the composting material during different stages.

Strain (domain/phylum)[a]	Sequence bp	Species	Accession number	Similarity (%)	No. of samplings[b]	Max (%)[c]	Stages[d]					
							RM	MES	THER	COO	MAT	FP
Bacteria												
Actinobacteria												
BT403	907	*Arthrobacter russicus*	NR_024783.1	99	11	1.5		+	+	+		+
AM946	929	*Brachybacterium paraconglomeratum*	JN6495995.1	98	8	24.0		+	+	+		
AM939	1176	*Corynebacterium casei*	DQ361013.1	99	13	14.2		+	+	+	+	+
AM929	1324	*Microbacterium gubbeenense*	EU863414.1	99	14	30.3	+	+	+	+	+	+
AM940	1305	*Microbacterium indicum*	NR_042459.1	96	8	9.3		+	+	+		+
Firmicutes												
BM7	929	*Bacillus licheniformis*	EF472268.1	100	10	9.6	+	+	+	+		+
AT595	784	*Bacillus licheniformis*	EU650317.1	99	11	7.1	+	+	+	+	+	+
BT309	902	*Bacillus licheniformis*	HM753625.1	98	18	48.5	+	+	+	+	+	
BT342	1353	*Bacillus licheniformis*	KC441778.1	99	12	46.7		+	+	+		+
BT315	928	*Bacillus subtilis*	FJ969738.1	99	14	5.9	+	+	+	+	+	
AM928	1310	*Bacillus subtilis*	JQ403532.1	99	17	18.5	+	+	+	+	+	+
AT636	1397	*Bacillus thermoamylovorans*	AB360815.1	99	8	12.7		+	+	+		+
BM24	916	*Brevibacterium halotolerans*	KC967073.1	99	11	11.0	+	+	+	+	+	+
BT343	952	*Ureibacillus thermosphaericus*	AB300774.1	99	10	4.7		+	+	+	+	
Proteobacteria												
BT378	1280	*Chelatococcus daeguensis*	HM000004.1	99	8	28.8		+	+	+		
BT352	1304	*Pseudoxanthomonas taiwanensis*	AB681369.1	99	12	40.3		+	+	+		
Fungi												
Ascomycota												
FT1445	629	*Aspergillus fumigatus*	HQ026746.1	99	8	40.0		+	+	+	+	
FM1217	798	*Candida mycetangii*	FJ381698.1	94	8	15.7	+	+	+			
FM1298	595	*Cladosporium lignicola*	AF393709.2	99	8	2.2		+	+		+	
FM1238	670	*Gibellulopsis nigrescens*	HE972037.1	100	16	25.2	+	+	+	+	+	
FM1377	444	*Ochrocladosporium frigidarii*	FJ755255.1	97	9	3.3		+		+		
FM1245	557	*Plectosphaerella cucumerina*	EU594566.1	99	10	11.5	+	+	+	+		
FM1274	659	*Scopulariopsis brevicaulis*	EU436681.1	98	18	59.9	+	+	+	+	+	+
FM1258	585	*Scopulariopsis brevicaulis*	EU821476.1	99	8	26.0	+	+	+		+	
FM1391	349	*Scopulariopsis brevicaulis*	KC311514.1	98	8	29.3		+	+	+		

[a] Bacteria 16S rRNA genes amplified; fungi, internal transcribed spacer (ITS1/ITS4) amplified.
[b] Number of samplings in which isolate was detected.
[c] Maximum percentage ratio of isolate (isolate count/total group count).
[d] Stages in which isolate was detected (+) (see Table 1 for sample characteristics): RM, raw material; MES, mesophile; THER, thermophile; COO, cooling; MAT, maturation; FP, final product.

Scopulariopsis. Several species of *Cladosporium* has been reported in composting (Ryckeboer et al. 2003; Anastasi et al., 2005) but never *Cladosporium lignicola*. This genus along with *Aspergillus* are regarded as the most common fungi in composting materials, due to their thermotolerance and/or capacity to degrade a wide range of organic waste (Anastasi et al., 2005). In particular, *Aspergillus fumigatus* is a well-known thermotolerant or thermophilic fungus (Anastasi et al., 2005; Ryckeboer et al., 2003). *Scopulariopsis brevicaulis* and *Gibellulopsis nigrescens* (previously named *Verticillium nigrescens*) have been earlier isolated from compost from different materials at mesophilic stage (Ryckeboer et al., 2003), while in the present work different strains of these species were detected in all stages. The closest relative to strain FM1217 was the yeast *Candida mycetangii* (Fig. 2) with a sequence similarity value of 94%, which is below the threshold level (e.g., 97% similarity) to consider the identification reliable. Consequently, isolate FM1217 deserves an in depth study to determine its correct identification. *Ochrocladosporium frigidarii* was named after its collection site, within a cooled incubation room (Crous et al., 2007). This may relate to the low thermal tolerance of the isolate FM1377 that matched with such a species. FM1377 was the unique representative of the selection that was not detected at thermophilic stages. Although this fungus is a cladosporium-like taxon, which is a naturally-occurring fungus in compost, this species has not been earlier reported in this ecosystem. *Plectosphaerella cucumerina*, the closest neighbor of isolate FM1245, is a widely spread fungus that has been found in mesophilic stage of composting mostly under the name of *Fusarium tabacinum* (Ryckeboer et al., 2003). This fungus has been reported to be a potential biological control agent against nematodes (Atkins et al., 2003).

These results provide new insights on the identity of culturable microorganisms that persists during all stages of lignocellulosic materials composting. Additionally, the selected isolates being ubiquitous in distribution are supposed to be highly successful at surviving in a wide range of environmental conditions, and to have great plasticity and physiological versatility. These are desirable qualities of microorganisms in several applications such as composting bioaugmentation, enzyme production or bioremediation that require further investigation.

3.2. Biotechnologically relevant microorganisms

The whole collection of isolates from compost (1457 isolates) were tested for the expression of ligninolytic, cellulolytic, hemicellulolytic, amylolytic, proteolytic, lipolytic, ammonifying and phosphate-solubilizing activities. Isolates exhibiting more than six activities were selected. Additionally, isolates capable of hydrolyzing lignocellulose components and/or bearing thermal tolerance were also included in the selection. According to these criteria 30 isolates (16 fungi and 14 bacteria) exhibited a range of metabolic activities and physiological capabilities that make them potentially suitable for a wide variety of applications, including enzyme production, composting improvement or compost bioaugmentation. The activities shown by isolates along with identification results

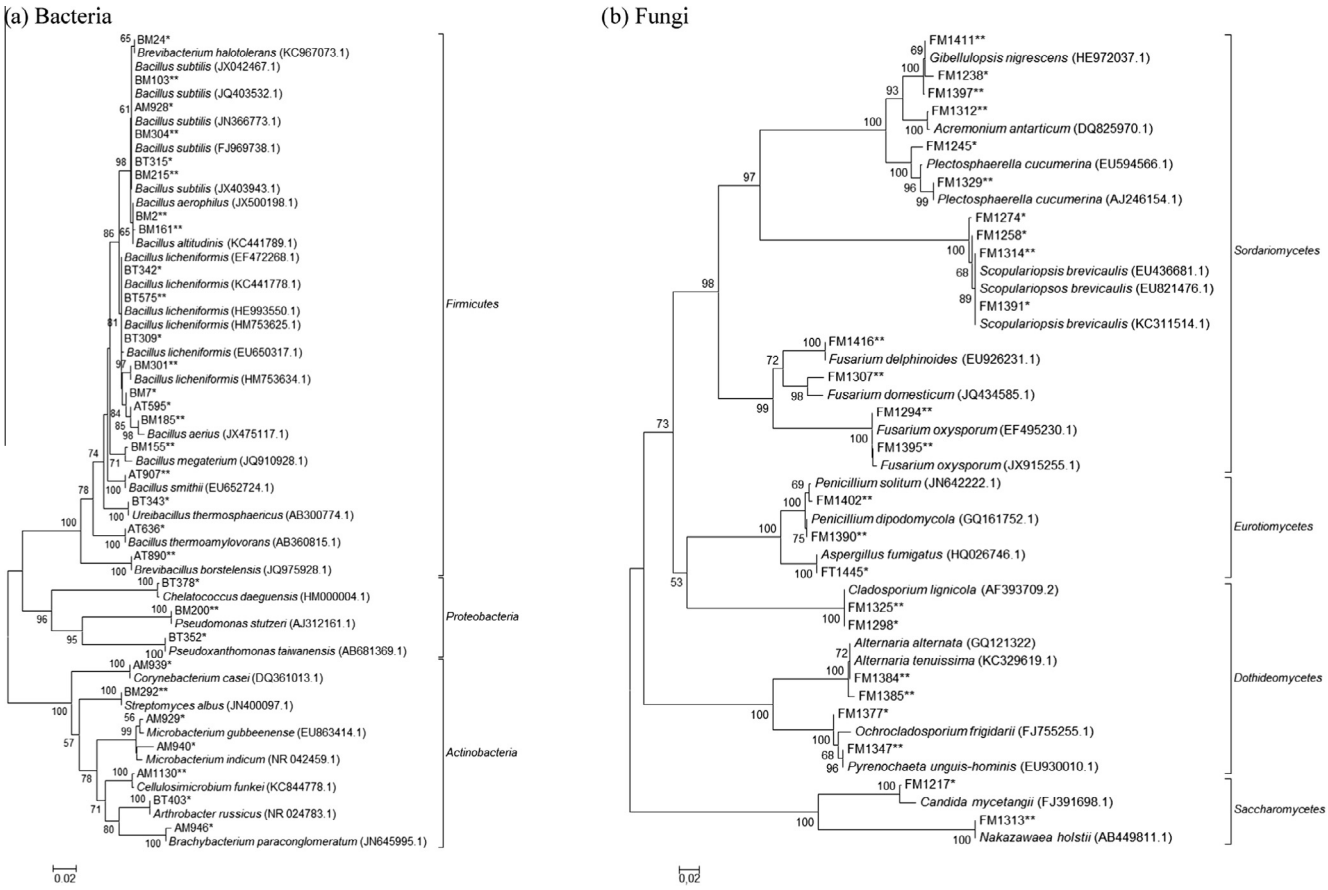

Fig. 2. Neighbour-joining phylogenetic tree based on (a) 16S rRNA gene sequences for bacterial isolates and (b) internal transcribed spacer (ITS1/ITS4) for fungal isolates selected from composting. The data of closely related sequences were recovered from GenBank and included in the tree. Accession numbers of the NCBI database are given in brackets. Sequences of persistent microorganisms are marked by (*) (see Table 2). Sequences of biotechnologically relevant microorganisms are marked by (**) (see Table 3). Phyla are indicated to the right of the group for bacteria and the classes for fungi (all fungi pertained to Phylum *Ascomycota*). The tree was constructed using MEGA with the Jukes–Cantor method for distance matrix calculations and the neighbor-joining method for tree design. Numbers at the nodes indicate levels of bootstrap support based on a neighbour joining analysis of 1000 resampled datasets; only values above 50% are given. The scale bar represents 0.02 substitutions per nucleotide position.

and their phylogenetic position are shown in Table 3 and Fig. 2, respectively. Most selected bacterial isolates belonged to *Firmicutes* (*Bacillus* and *Brevibacillus*), only one was a *Proteobacteria* (*Pseudomonas*) and two affiliated with *Actinobacteria* (*Streptomyces* and *Cellulosimicrobium*). The range of fungal genera that meet the selection criteria was slightly wider than that of bacteria. Nine fungal genera, all affiliated to *Ascomycota*, were among identified fungal isolates, including the filamentous fungi *Alternaria*, *Cladosporium*, *Pyrenochaeta*, *Penicillium*, *Acremonium*, *Fusarium*, *Plectosphaerella* and *Scopulariopsis*, and the yeast *Nakazawea.* The species *B. licheniformis*, *C. lignicola, G. nigrescens, P. cucumerina* and *S. brevicaulis*, were also detected as persistent species during composting (Table 2). As stated earlier, some of these species are described in composting for the first time. In addition, the fungal species *Pyrenochaeta unguis-hominis*, *Penicillium dipodomycol*a, *Acremonium antarcticum* and *Fusarium delphinoides*, and the yeast *Nakazawea holstii*, have not been earlier reported in this ecosystem. The genera *Bacillus* and *Fusarium* dominated among isolates showing the highest diversity of activities.

All selected isolates exhibited ammonifying activity which was linked to the presence of proteolytic activity, since most isolates (25 isolates) also expressed this capability. Proteins are degraded to amino acids (proteolysis) and then to inorganic NH_4^+, by heterotrophic ammonifying microorganisms. Both activities would lead to the release of total ammoniacal nitrogen during composting. Although the specific composition of both communities has not been reported for composting ecosystems it is well-know that most soil microorganisms express both activities (Vranova et al., 2013).

Among the activities related to polysaccharide hydrolysis, hemicellulolytic (28 isolates) and amylolytic (20 isolates) were the most widespread within the selected isolates, while less than 50% of the isolates were cellulolytic (14 isolates) or pectinolytic (12 isolates). These activities have received a great deal of attention due to the potential application in sugar, textile, brewing, pharmaceuticals and in the food, feed, pulp and paper industries (Olson et al., 2012). Noteworthy, several strains exhibited potential for degrading all polysaccharides e.g. *P. cucumerina*, *G. nigrescens*, *Fusarium oxysporum*, *Fusarium domesticum*, *F. delphinoides* and *P. unguis-hominis.* These microorganisms can be good candidates for consolidated bioprocesses, the conversion of lignocellulose into desired products in one step without added enzymes (Olson et al., 2012).

Ligninolysis was the less common activity among isolates, only 3 bacterial isolates identified as *Streptomyces albus* (BM292), *Bacillus smithii* (AT907) and *Brevibacillus borstelensis* (AT890) and the fungus *C. lignicola* (FM1325) expressed this activity. It is well-known that lignin degrading capability is mainly attributed to fungi, which use oxidative extracellular mechanisms to break down the lignin polymer. Ligninolytic bacteria are less well studied, but several examples have been found among proteobacteria (e.g., *Pseudomonas* sp.) and mainly actinobacteria (Bugg et al.,

Table 3
Biotechnologically relevant microorganisms isolated from the composting material.

Strain (domain/phylum)[a]	Sequence bp	Species	Accession number	Similarity (%)	Activity[b]								
					A	Pc	H	C	Lg	Lp	Am	Pr	Ps
Bacteria													
Actinobacteria													
AM1130	1345	*Cellulosimicrobium funkei*	KC844778.1	99	+	–	+	–	–	–	+	+	–
BM292	1094	*Streptomyces albus*	JN400097.1	99	+	–	+	–	+	+	+	–	+
Firmicutes													
BM185	996	*Bacillus aerius*	JX475117.1	99	+	–	+	–	–	+	+	+	+
BM161	849	*Bacillus aerophilus*	JX500198.1	98	+	–	+	–	–	+	+	+	+
BM2	1373	*Bacillus altitudinis*	KC441789.1	100	+	+	+	–	–	+	+	+	+
BT575	1453	*Bacillus licheniformis*	HE993550.1	100	+	+	+	–	–	–	+	+	–
BM301	1389	*Bacillus licheniformis*	HM753634.1	99	+	–	+	–	–	+	+	+	+
BM155	528	*Bacillus megaterium*	JQ910928.1	99	+	–	+	–	–	+	+	+	+
AT907	1348	*Bacillus smithii*	EU652724.1	99	+	–	–	–	+	–	+	–	–
BM304	1288	*Bacillus subtilis*	JN366773.1	99	+	–	+	–	–	+	+	+	+
BM103	1378	*Bacillus subtilis*	JX042467.1	99	+	–	+	–	–	+	+	+	+
BM215	858	*Bacillus subtilis*	JX403943.1	99	+	–	+	–	–	+	+	+	+
AT890	1352	*Brevibacillus borstelensis*	JQ975928.1	99	–	–	–	–	+	–	+	+	–
Proteobacteria													
BM200	1355	*Pseudomonas stutzeri*	AJ312161.1	98	–	+	+	–	–	+	+	+	+
Fungi													
Ascomycota													
FM1312	576	*Acremonium antarcticum*	DQ825970.1	99	–	+	+	+	–	+	+	+	–
FM1384	598	*Alternaria alternata*	GQ121322.2	100	–	–	+	+	–	+	+	+	–
FM1385	438	*Alternaria tenuissima*	KC329619.1	99	+	+	+	–	–	+	+	+	–
FM1325	597	*Cladosporium lignicola*	AF393702.2	100	–	–	+	+	+	–	+	–	–
FM1416	602	*Fusarium delphinoides*	EU926231.1	100	+	+	+	+	–	+	+	+	–
FM1307	595	*Fusarium domesticum*	JQ434585.1	96	+	+	+	+	–	–	+	–	–
FM1294	550	*Fusarium oxysporum*	EF495230.1	99	–	–	+	+	–	+	+	+	–
FM1395	585	*Fusarium oxysporum*	JX915255.1	99	–	–	+	+	–	+	+	+	–
FM1397	599	*Gibellulopsis nigrescens*	HE972037.1	99	+	+	+	+	–	+	+	+	–
FM1411	599	*Gibellulopsis nigrescens*	HE972037.1	96	+	+	+	+	–	–	+	+	–
FM1313	678	*Nakazawea holstii*	AB449811.1	100	–	+	+	+	–	+	+	+	–
FM1390	618	*Penicillium dipodomycola*	GQ161752.1	99	+	–	+	–	–	+	+	+	+
FM1402	626	*Penicillium solitum*	JN642222.1	99	–	–	+	+	–	+	+	+	–
FM1329	563	*Plectosphaerella cucumerina*	AJ246154.1	99	+	+	+	+	–	–	+	–	–
FM1347	588	*Pyrenochaeta unguis-hominis*	EU930010.1	99	+	+	+	+	–	+	+	+	–
FM1314	612	*Scopulariopsis brevicaulis*	EU436681.1	99	–	–	+	+	–	+	+	+	–

[a] Bacteria 16S rRNA genes amplified; fungi, internal transcribed spacer (ITS1/ITS4) amplified.
[b] Biotechnologically relevant activities analyzed: A, amylolytic; Pc, pectinolytic; H, hemicellulolytic; C, cellulolytic; Lg, ligninolytic; Lp, lipolytic; Am, ammonifying; Pr, proteolytic; Ps, phosphate solubilization.

2011). However, the number of bacteria showing ligninolytic potential independently from lignin utilization by the decolorization of synthetic lignin-like dyes is wider. As in the present work, this activity has been demonstrated in several species of the genus *Bacillus* (including *B. smithii*) (Rajasundari and Murugesan, 2011).

Lipolytic activity was detected in 22 isolates, including 10 bacteria pertaining to the genera *Bacillus, Pseudomonas* and *Streptomyces*, and 12 fungal species of the genera *Fusarium*, *Alternaria*, *Penicillium*, *Scopulariopsis*, *Acremonium* and *Pyrenochaeta*. These species, except *Pyrenochaeta*, has been earlier reported as lipase producers (Hasan et al., 2006). Lipases have many applications, such as organic syntheses, hydrolysis of fats and oils, modification of fats, flavor enhancement in food processing, resolution of racemic mixtures and chemical analyses (Hasan et al., 2006).

Phosphate-solubilizing microorganisms (11 isolates) mostly belonged to *Bacillus*, also two isolates were identified as *S. albus* and *Pseudomonas stutzeri*, and only one fungus, *P. dipodomycola*, exhibited this activity. Although both bacteria and fungi are known to solubilize phosphorous, bacteria are more effective in phosphorus solubilization than fungi. The genera *Bacillus* and *Pseudomonas* are among the most frequently associated to this activity, although there are also some reports on *Streptomyces* and *Penicillium* (Behera et al., 2014). These microorganisms may find several agronomic applications such as biofertilizer, plant growth promotion and antagonist of phytopathogen (Behera et al., 2014).

This study led to the isolation of microorganisms from the composting exhibiting a wide range of metabolic activities of potential biotechnological interest. Future studies are required to determine the exact nature of the activity undertaken by each of these organisms.

3.3. Biotransformation of organic matter by selected isolates

The metabolic capabilities detected in selected isolates (Table 3) are not necessarily expressed by the microorganisms during the composting; consequently, the expected modification of lignocellulose, protein or lipids by the specific enzymes (e.g. cellulase, hemicellulase, protease, lipase) may not take place once they are used as inoculants for composting. In addition, when an inoculated microorganism is brought into contact with non-sterile lignocellulosic substrata, which happens under composting conditions, the inoculum has to interact with the native composting microorganisms. Depending on the competitive ability of the inoculum and the composting microorganisms, the latter may restrain growth and activities of the inoculated microorganism, therefore inhibiting its degradation capability. Thus, in order to determine the potential application of the selected isolates as inoculants for composting, a two phases experiment was performed.

First, the isolates were inoculated on lignocellulosic material and the increase of SOC and C_{mic} was calculated as described

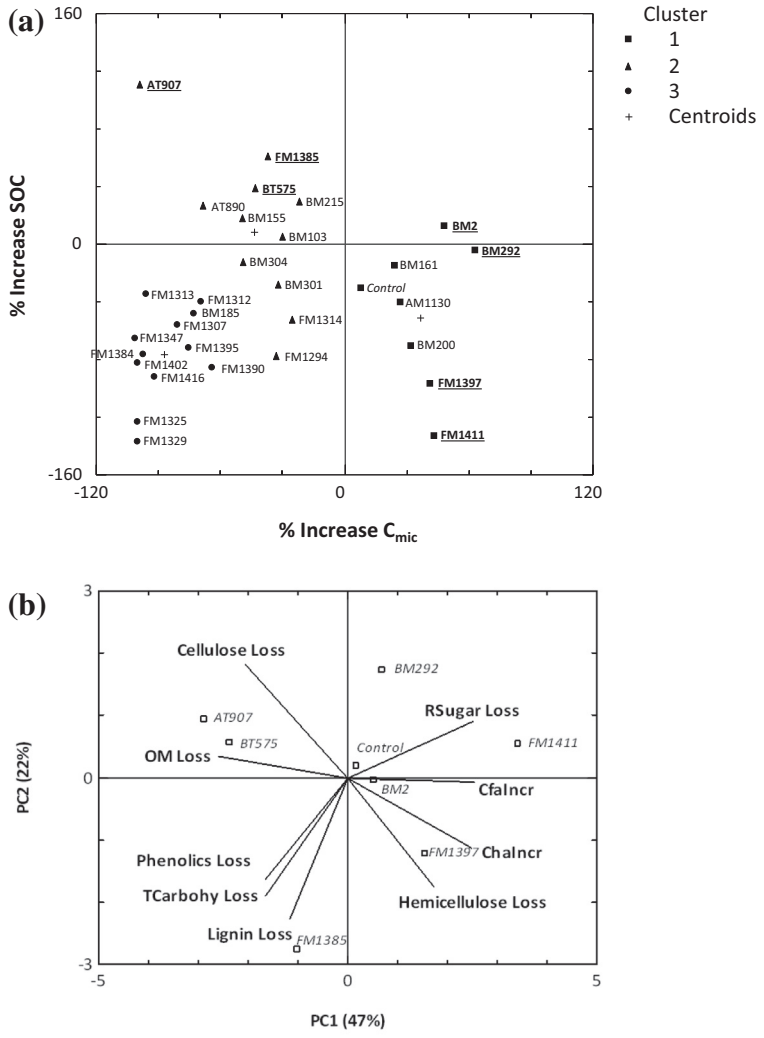

Fig. 3. (a) Cluster scatterplot (Ward's method, squared Euclidean) of treatment of lignocellulosic material with inocula (control, non-inoculated material) according to capability to increase soluble organic carbon (% Increase SOC) or microbial biomass carbon (% Increase C_{mic}). Inocula selected are bold marked and underlined. (b) Principal component analysis of data showing loadings plots for treatment with selected isolates. Percent variability explained by each principal component (PC) is shown in parentheses. The variables used in the analyses are displayed: % loss of total organic matter (OM Loss), lignocellulose fractions (Cellulose, Hemicellulose and Lignin Loss), total carbohydrates (TCarbohy Loss), soluble reducing sugar (RSugar Loss) and phenolic acids (Phenolics Loss); as well as those revealing *de novo* formation by humification, i.e. fulvic-like, (CfaIncr) and humic-like (ChaIncr) fractions % increase.

earlier. On the basis of the wide capability to breakdown polymeric compounds exhibited by the selected isolates, it is expected they would release monomeric compounds from the lignocellulosic matrix (increasing SOC) and promote microbial growth (increasing C_{mic}) as a consequence. A cluster analysis was carried out on the data obtained (see Supplementary material, Table S1) that grouped treatments into three clusters depending on their capability to increase SOC and C_{mic} (Fig. 3a). Seven inocula (2 fungi and 5 bacteria) and the control (non-inoculated) were grouped in cluster 1, comprising treatments that lead to an increase in C_{mic}. Cluster 2 mainly included treatments (1 fungus and 6 bacteria) that led to an increase in SOC but did not promote microbial growth (C_{mic}). The remaining treatments (11 fungi and 1 bacterium) grouped in cluster 3 and did not increase SOC or C_{mic}. Surprisingly, only treatment with *Bacillus altitudinis* BM2 (cluster 1) caused, as expected, an increase of both SOC and C_{mic}. In a composting process SOC fluctuates at the early stages because it is used for microbial growth as it is released, but at the late stages (cooling and maturation) it decreases as organic matter stabilizes (López-González et al., 2013). The biotransformation process developed in the present work mimic the mesophilic stage of composting, thus the treatments that could benefit the process are those that lead to the release of soluble organic compounds. Even if they do not stimulate the growth at this stage, it could be expected to have a biostimulating impact in the further stages. Also, treatments that promote microbial growth are desirable because they cause a spread out of the bioaugmentation intended.

According to the previous results, seven inocula that caused wider increase in SOC (*S. albus* BM292, *B. altitudinis* BM2, *G. nigrescens* FM1397 and *G. nigrescens* FM1411) or C_{mic} (*B. licheniformis* BT575, *B. smithii* AT907 and *Alternaria tenuissima* FM1385) were selected for the following experimental series. These microorganisms were inoculated in lignocellulosic material and relevant compounds that inform on the organic matter dynamics were analyzed and divided into two groups. The first group included all compounds that are expected to be degraded (or loss) during composting by mineralization or to be incorporated as components of microbial biomass: i.e. total organic matter (OM loss), lignocellulose fractions (cellulose, hemicellulose and lignin loss), polymeric and soluble carbohydrates (total carbohydrates and reducing sugar loss) and phenolic acids (phenolic acid loss). In the second group those revealing *de novo* formation by humification (fulvic-like

and humic-like fractions increase) were analyzed and their increase was calculated. A principal component analysis (PCA) was applied to the data matrix obtained (see Supplementary material, Table S2). The first two principal components (PC1/PC2) explained 69% of the variability. The scoring coefficients of the eight treatments (seven selected inocula and non-inoculated control) were used to draw their principal component analysis chart and the variations of composting parameters were examined (Fig. 3b). The most influential variables in PC1 (47% of variance) were organic matter and cellulose loss with negative load; and humic-like and fulvic-like acids increase with positive load. Accordingly, PC1 characterized humification and it also produced a separation between treatments with inocula. Thus, *B. smithii* AT907, *B. licheniformis* BT575 have more influence on breakdown of polymeric compounds (lignin and cellulose), while *S. albus* BM292 and *G. nigrescens* FM1397 and FM1411 stimulate the repolymerization of organic matter into humic and fulvic-like substances along with hemicellulose breakdown. The non-inoculated (Control) and *B. altitudinis* BM2 treatments did not lead to significant variations on the compounds evaluated. The second component (PC2) accounted for 22% of the total variance; it was characterized by high positive loading (>0.40 threshold) for cellulose loss and negative loads for lignin, phenolic acids, total carbohydrates and hemicellulose loss. Noticeably, this component gave opposite loads between lignin loss and cellulose loss which may relate to the known restriction the former component causes in the degradation of cellulose (Turner et al., 2007). In this sense, the fungus *A. tenuissima* FM1385 was more active in lignin degradation while *S. albus* BM292 and *B. smithii* AT907 had more influence on the degradation of cellulose.

The results obtained confirm that the screening procedure allowed obtaining microorganisms with great potential as inoculants for composting improvement. The microorganisms selected had the capability to breakdown a wide range of polymeric compounds, whose activity was confirmed by the biotransformation pattern they caused in lignocellulosic material. This was different depending on the inoculum with some of them being more active in the degradation of specific polymers and others in humification processes. It remains to be known whether the behavior exhibited by the isolates either as pure cultures or in a designed mixed consortium could be translated to a full scale composting process. The interactions among isolates in a mixed consortium also require further investigation.

4. Conclusions

The persistent taxa and functional groups of microorganisms during lignocellulose-based composting were analyzed. Some species of persistent isolates are detected for the first time in this ecosystem. Several bacterial and fungal isolates exhibited metabolic capabilities that may find many biotechnological applications. Seven isolates showed a high potential as inoculants for composting because of its capability to modify organic matter. This study contributes to catalogue specific microorganisms in composting ecosystem, reaffirms the potential of the composting as a source of interesting and useful microorganisms, and provides additional information to support future research about the biotechnological potential of these microorganisms.

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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.biortech.2014.03.145.

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