




Article

Enzymatic Potential of Bacteria and Fungi Isolates from the Sewage Sludge Composting Process

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Featured Application: Screening of suitable microorganisms adapted to environmental conditions are the challenges for the optimization of biotechnological processes in the current and near future. Some of the isolated microorganisms in this study possess biotechnological desirable features which could be employed in different processes, such as biorefinery, bioremediation, or in the food industry.

Abstract: The aim of this study was the isolation and characterisation of the fungi and bacteria during the composting process of sewage sludge under a semipermeable membrane system at full scale, in order to find isolates with enzymatic activities of biotechnological interest. A total of 40 fungi were isolated and enzymatically analysed. Fungal culture showed a predominance of members of Ascomycota and Basidiomycota division and some representatives of Mucoromycotina subdivision. Some noticeable fungi isolated during the mesophilic and thermophilic phase were *Aspergillus*, *Circinella*, and *Talaromyces*. During the maturation phase, some lignin modifying enzyme producers, like *Purpureocillium*, *Thielavia*, *Bjerkandera*, or *Dichotomyces*, were found. Within this group, *Thielavia* and *Bjerkandera* showed high activity with production of laccases and peroxidases. In the bacterial culture, a total of 128 strains were selected and enzymatically analysed. Bacillales, Actinomycetales, Pseudomonadales, and Lactobacillales were the orders most represented in culture-bacteria. *Bacillus pumilus*, *B. stratosphericus*, *B. safensis*, and *Pseudomonas formosensis* were the species most efficient in enzyme production, particularly peroxidases, polyphenol oxidases ammonifying activity, and amylases. These results showed that sewage sludge composting piles could represent a source of microorganisms which have adapted to adverse conditions.

Keywords: bacteria; composting; enzymes; fungi; sewage sludge

1. Introduction

Composting is a common process to stabilise sewage sludge, which allows the revalorisation of this waste as amendment. During composting, microorganisms use both extracellular and intracellular enzymes to hydrolyse organic matter and to transform complex compounds into simple molecules that can be assimilated and finally mineralised [1,2]. Enzyme activities are considered as sensor or indicator of the degradation, since they are an indirect measurement of the availability of nutrients, microbial activity, and the physical-chemical conditions. These properties vary depending on the state

of the compost, on the transformation of organic matter, and on the functional diversity [3–6]. A wide variety of fungi and bacteria are able to break and oxidise macromolecules using a set of hydrolytic, oxidative lyase, and transferase enzymes [7]. In addition, dehydrogenase, β -glucosidase, proteases, phosphatases, and arylsulfatase are the main enzymes proposed to monitor the activity of microbial communities [8].

The associated microbiota during composting is widely diverse and its ecology is extremely complex. Different microbial activities occur depending on the physical-chemical and nutritional conditions prevailing at each moment and throughout the process the microorganisms themselves change the physical-chemical properties. The conditions of the process, like the autochthonous microbiota, temperature, and moisture, or the composition of the sewage sludge, make the pile an interesting ecosystem and a singular environment to search for microorganisms with abilities to produce enzymes with biotechnological applications. Among them, the ones that stand out are those that can be applied in the biosynthesis processes, food industry, biorefineries, and textile industries, like hemicellulases, pectinases, oxidoreductases, laccases, and peroxidases.

Previous studies on microbial diversity have shown that many of the predominant taxa in the different phases of composting possess high capacity to produce enzymes. The study performed by Jurado et al. [9] reported that genera like *Alternaria*, *Cladosporium*, *Penicillium*, *Acremonium*, *Fusarium*, and *Scopulariopsis* for fungi and *Bacillus*, *Brevibacillus*, *Pseudomonas*, *Streptomyces*, and *Cellulosimicrobium* for bacteria, isolated from lignocellulosic waste composting, were microorganisms with a high potential for enzyme production [9]. However, as far as we know, there have been no studies about the potential of enzyme production in microorganisms isolated from sewage sludge composting.

In this context, the aim of this study was to isolate fungi and bacteria from different phases of the sewage sludge composting pile built at full-scale and with a semipermeable membrane system, in order to assess the capacity of these isolates as candidates in the production of extracellular enzymes with biotechnological applications. This preliminary study could provide the first step in an interesting approach to find microorganisms with metabolic abilities to degrade micropollutants present in sewage sludge. To reach this objective, a screening of qualitative production of hydrolytic and oxidative enzymes was performed in a collection of isolates from a sewage sludge composting pile.

2. Materials and Methods

2.1. Full-Scale Composting Pile and Sampling

The sewage sludge composting pile was performed at industrial scale in the Biosolid Plant of “Biomasa del Guadalquivir”. This pile was located in Vegas del Genil, Granada (37.160791–3.723879) and it was built using a semipermeable film to cover the pile and a ventilator system, as previously described by Robledo-Mahón et al. [10]. The samples were taken at 5 different depths of the pile (0, 25, 50, 75 and 100 cm) and mixed to create a single sample following the composite sample procedure. The samples used in the isolation of microorganisms were from sewage sludge, bulking agent (both as starting material), and at different times: 0 days (initial mixture), 1, 2, 5, 8, 14, 30 (under cover phase), 40, 70 and 120 days (maturation phase).

2.2. Isolation and Maintenance of Bacteria and Fungi Collection

To isolate the microbiota by culture-dependent techniques from composting samples, 10-fold serial dilutions were performed from 1 g of each sample diluted in 9 mL of sterile saline solution (0.9% NaCl, Carlos Erba Reagents Srl, Barcelona, Spain). Different dilutions were performed (10^1 – 10^6) and 0.1 mL of each dilution was spread on Petri dishes containing the appropriate medium. Malt extract agar (MEA, VWR-Prolabo Chemicals, Barcelona, Spain) was used for fungi and Trypticase Soya Agar medium (TSA, Thermo Fisher Scientific-Oxoid, Madrid, Spain) for bacteria. In MEA, 50 $\mu\text{g L}^{-1}$ of streptomycin and tetracycline were added to inhibit bacterial growth [11,12]. Each dilution was performed in triplicate. The dishes were incubated for 96 h at 28 °C and 55 °C for fungi and 48 h at 30 °C

and 55 °C for bacteria, in order to distinguish between mesophilic and thermophilic microorganisms. After incubation time, different colonies were selected according to morphotype features and persistence in replicates. Each colony was transferred to a new plate containing the appropriate medium. For long storage of pure cultures, cryovials were prepared using 60% and 80% of glycerol for fungi and bacteria, respectively; with two replicates at −20 °C and −80 °C.

2.3. Determination of Hydrolytic Enzymes

Proteolytic, amylolytic, phosphatase solubilising, lipolytic, pectinolytic, hemicellulolytic, cellulolytic, and ammonifying activity were determined. The method used was based on the colorimetric changes. Petri dishes containing Potato Dextrose Agar (PDA) for fungi and TSA for bacteria and the corresponding substrate were used. Ammonifying activity was determined in 9 mL liquid medium using Potato Dextrose Broth (PDB) and Tryptone Soya Broth (TSB) for fungi and bacteria, respectively. The inoculums of fungi were small pieces of mycelium of 0.5 × 0.5 mm, taken from PDA Petri dishes, incubated approximately 96 h at 28 °C. The inoculums of bacteria consisted of 30 µL of overnighed culture at 30 °C.

Briefly, the substrates used by each enzyme were 1% of sodium caseinate (ACROS Organics, Madrid, Spain) for proteolytic activity [13]; 1% of starch (Scharlab SI, Barcelona, Spain) for amylolytic activity [14]; 2.5% of phosphate tricalcium (PanReac Química S.L.U, Barcelona, Spain) for soluble phosphate solubilizing activity [15]; 1% of tributiryn (Merck) for lipolytic activity [16]; 0.5% of pectin (Sigma-Aldrich, Madrid, Spain) for pectinolytic activity [17]; 0.5% of xylan for hemicellulose activity [18]; 0.5% of microcrystalline cellulose and 0.005% of nigrosin for cellulolytic activity [19], and 0.02% of asparagine (Sigma-Aldrich) for ammonifying activity [20]. Incubation temperatures were 28 °C for fungi and 30 °C for bacteria. Incubation time varied depending on the enzymatic activity analysed. Thus, for proteolytic, amylolytic, and phosphate enzyme activity, the microorganisms were incubated 3 days; for lipolytic, pectinolytic, and hemicellulolytic activity 5 days; for cellulolytic activity incubation 7 to 10 days; and for ammonifying activity 15 days. Each determination was performed in triplicate. Positive results were considered after the appearance of a clear halo around the colony growth after the incubation time. In the case of amylolytic activity, it was necessary to add 1–2 mL of iodine solution to decipher a clear halo around the colony. The results of pectinolytic activity were determined by the addition of 2 mL of 0.05% red ruthenium dye solution. This was incubated for 1 h at room temperature and washed with distilled water. A clear halo around the colony was considered to be a positive result. In the case of ammonifying activity, the addition of 1 mL Neßler reagent (Sigma-Aldrich) and observing the formation of orange precipitate was necessary. More details are described in Table S1 of the Supplementary Material.

2.4. Spot Test for Determining Oxidative Enzymes

The oxidative enzymes assessed in this study were tyrosinase, laccase, peroxidase, and polyphenol oxidase, following the spot test methodology described by Gramss et al. [21]. For that, fungal and bacterial isolates were previously grown in PDA and TSA, respectively. Petri dishes were incubated for 96 h for fungi and 24 h for bacteria. The colonized Petri dishes were used for the addition of different solutions by droplets at the edge of each colony: 0.1 M *p*-cresol for tyrosinase, 0.1 M 1-naphthol for laccase, 0.5% of pyrogallol and 0.5% of hydrogen peroxide for peroxidase, and 0.5% of pyrogallol for polyphenol oxidase. Each determination was performed in triplicate. After droplet addition, Petri dishes were incubated 24 h at 28 °C and 36 °C for fungi and bacteria, respectively, to observe colorimetric changes. Changes in the colour of the edge of the colony were considered positive results being yellow-red for tyrosinase, blue-purple for laccase, and brown for peroxidase and polyphenol oxidase. Table S2 supplies detailed information about the preparation and interpretation of the results.

2.5. Identification of Fungi and Bacteria by Molecular Techniques

The phylogenetic identification was performed in the Department of Nucleic Acids of the instrumental techniques laboratory of the University of León (Spain). DNA isolation was performed using PrepMan™ Ultra (Applied Biosystems, Madrid, Spain) for each selected isolate. The amplification of genes of interest was carried out by PCR in 50 µL of reaction with DreamTaq DNA polymerase Kit (Thermo Fisher Scientific, Madrid, Spain) from 1 µL of DNA and 20 pmol of each primer. For fungi, the pairs of primers used were ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [22], to amplify the internal transcriber spacer ITS1 and ITS2, that includes 5.8S rRNA genes. Also, the pair of primers NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') of the 28S rRNA gene region were used for yeasts. The pairs of primers used for bacteria were 27 F (5'-AGAGTTTGATCMTGGCTCAG-3') and 699R (5'-RGGGTTGCGCTCGTT-3') and BA-RF (5'-GACGATCATYTWGGAAACCG-3') and BA-RR (5'-GGNGTYTCRATYGGACACAT-3') [23] to amplify different regions of the bacterial 16S rRNA gene. A GeneAmp® PCR System 2700 (Applied Biosystems Thermocycler, Madrid, Spain) was used. The standard PCR program was performed with initial denaturing at 95 °C for 5 min for 35 cycles (95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min) with a final extension at 72 °C for 10 min. PCR products were visualised in 1% agarose gel and purified using NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel GmbH & Co., Düren, Germany) according to manufacturer's instructions and were sequenced by BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Madrid, Spain) in capillary automatic sequencer ABI 3130xl (Applied Biosystems, Madrid, Spain). The consensus sequences obtained were edited using the program CHROMAS LITE v.2.0 (Chromas-Technelysium Pty Ltd., Brisbane, Australia) and compared with the sequences in GenBank database using the Basic Local Alignment Search Tool (National Center for Biotechnology Information, U.S. National Library of Medicine, Bethesda, MD, USA) software.

3. Results

3.1. Profile of Extracellular Enzymes Produced by Fungi and Bacteria Isolated during the Composting Process

A total of 105 fungal strains and 128 bacterial strains were initially isolated. However, in the case of fungi, only 40 could be maintained at laboratory conditions. Consequently, a total of 40 fungal and 128 bacterial strains were tested for hydrolytic and oxidative enzyme activities. The results obtained in the distribution of enzymatic activities are summarised in Figure 1. For fungi (Figure 1A), the most frequent secreted enzymes were amylase (72.5%), peroxidase (47.5%), polyphenol oxidase (42%), pectinase (42.5%), cellulase (32.5%), and protease (40%). In this case, the less frequent activities were tyrosinase and P-solubilising.

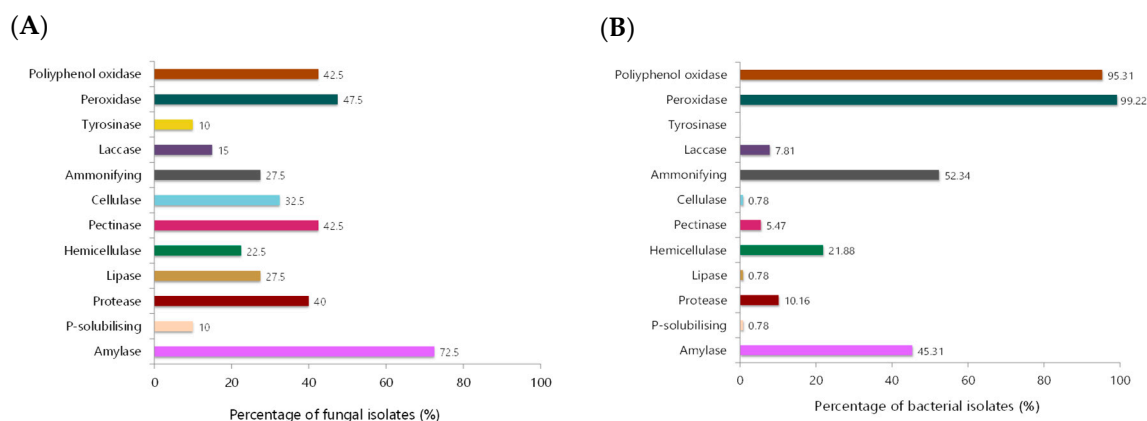


Figure 1. Percentage of enzymes produced by (A) fungal and (B) bacterial isolates.

In the case of bacteria (Figure 1B), the most frequent activity produced was polyphenol oxidase (95.3%) and peroxidase (99.2%). As well, ammonifying activity (52.3%) was present in more than a half of the bacteria isolated. Amylase was another frequent enzyme (45.3%) in bacterial isolates. The less common activities were cellulase, lipase, and P-solubilising.

The distribution of the analysed enzymes along time is shown in Figure 2. In the case of fungi (Figure 2A), amylolytic activity was predominant at the beginning of the process and in the maturation stage. An absence or decrease in the number of isolates producing enzymes between samples 2 and 8 days can be observed. From 14 days, there were more isolates producing enzymes like peroxidase, pectinase, hemicellulose, and tyrosinase, among others. Therefore, the fungal isolates from the initial part of the process and from the maturation stages showed a high number of extracellular enzyme activities.

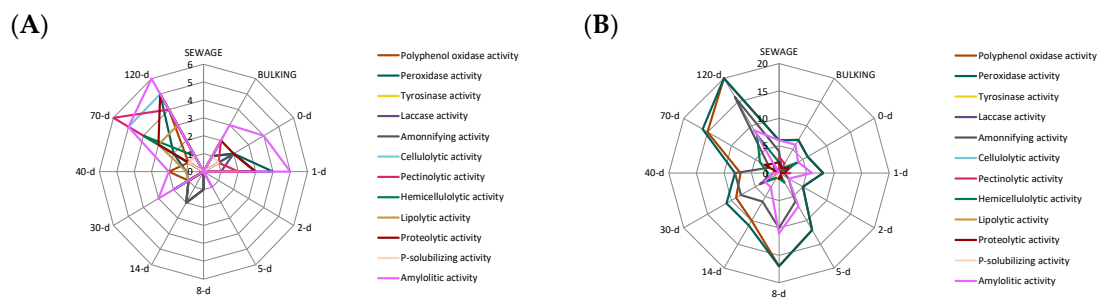


Figure 2. Number of extracellular enzymes produced by fungal (A) and bacterial (B) isolates in each composting sample.

Concerning bacterial isolates, the highest number of activities was found in the isolates belonging to 8, 70 and 120 days, being peroxidase and polyphenol oxidase (Figure 2B) followed by ammonifying enzymes and amylases as the predominant enzymes. The highest number of strains with peroxidase and polyphenol oxidase was at 120 followed by samples at 8 and 70 days. Lignocellulolytic activities like peroxidases and polyphenol oxidase were found in a high number of fungal and bacterial isolates, being mostly isolated from the maturation phase, corresponding to the 70 and 120 day samples.

3.2. Metabolic Characterisation and Phylogenetic Identification of Fungal Isolates

For fungal strains, the GenBank accession numbers for the ITS-region and for 28S rDNA sequences are MT60989-MT609917. In Figure 3, a detailed heat map with the activities produced by each fungal strain is provided. A total of 105 fungal strains were isolated during composting, but only 40 could be cultured for a long time. A total of 40 fungal strains were identified and metabolically characterised. The phylogenetic study shows high taxonomical diversity, since these isolates belonged to 23 different genera, of which most of them had a single representative. A clear succession was observed within this population with a change in the orders belonging to the early phase under semipermeable film (0, 1, 2, 5 and 8 days) corresponding to Sporidiobolales, Saccharomycetales, and Agaricales. During the advanced phase (15 and 30 days), Capnoidales, Trichosporonales, and Mucorales were the most predominant. In the maturation phase (70 and 120 days), these were Hypocreales, Sordariales, and Polyporales. However, the presence of Eurotiales and Microascales was consistent throughout the whole process.

Sample	Strain name	Phylogenetic identification	Hydrolytic				Lignocellulosic				Identity (%)	Access number (GenBank)	Order	
			Peptidolytic	Amylolytic	P-solubilizing Lipase	Amomnifying Tyrosinase	Pectinase	Hemicellulase	Cellulase	Laccase				Peroxidase
B	HM01	<i>Aspergillus tubingensis</i>	+	+	+	+	+	+	+	+	+	100	KP725001.1	Eurotiales
	HM02	<i>Byssoschlamys spectabilis</i>	+	+	+	+	+	+	+	+	+	100	KP725000.1	Eurotiales
	HM03	<i>Scopulariopsis brevicaulis</i>	+	+	+	+	+	+	+	+	+	100	LN850790.1	Microascales
	HM08	<i>Blastobotrys raffinifermentans</i>	+	+	+	+	+	+	+	+	+	100	KP131654.1	Saccharomycetales
	HM09	<i>Rhodospiridium kratochvilovae</i>	+	+	+	+	+	+	+	+	+	100	KC442276.1	Sporidiobolales
O	HM10	<i>Rhodotorula mucilaginosa</i>	+	+	+	+	+	+	+	+	+	94	LC125991.1	Sporidiobolales
	HM11	<i>Eurotium chevalieri</i>	+	+	+	+	+	+	+	+	+	99	HQ728257.1	Eurotiales
	HM12	<i>Scedosporium aurantiacum</i>	+	+	+	+	+	+	+	+	+	100	JQ690909.1	Microascales
	HM17	<i>Aspergillus tubingensis</i>	+	+	+	+	+	+	+	+	+	100	KP725001.1	Eurotiales
	HM18	<i>Hormographiella aspergillata</i>	+	+	+	+	+	+	+	+	+	100	KP132299.1	Agaricales
1	HM19	<i>Byssoschlamys spectabilis</i>	+	+	+	+	+	+	+	+	+	87	KP725000.1	Eurotiales
	HM22	<i>Emericella nidulans</i>	+	+	+	+	+	+	+	+	+	100	KC806056.1	Eurotiales
	HM23	<i>Tritiratum oryzae</i>	+	+	+	+	+	+	+	+	+	90	GQ329853.1	Mucorales
	HM28	<i>Talaromyces purpureogenus</i>	+	+	+	+	+	+	+	+	+	99	KU981069.1	Eurotiales
	HM29	<i>Aspergillus versicolor</i>	+	+	+	+	+	+	+	+	+	100	JX043092.1	Eurotiales
	HM30	<i>Aspergillus versicolor</i>	+	+	+	+	+	+	+	+	+	100	GU797139.1	Eurotiales
	HT04	<i>Lichtheimia ramosa</i>	+	+	+	+	+	+	+	+	+	98	GQ342877.1	Mucorales
5	HM38	<i>Byssoschlamys spectabilis</i>	+	+	+	+	+	+	+	+	87	KP725000.1	Eurotiales	
8	HM40	<i>Cladosporium cladosporioides</i>	+	+	+	+	+	+	+	+	100	HQ829120.1	Capnodiales	
14	HM41	<i>Cladosporium cladosporioides</i>	+	+	+	+	+	+	+	+	100	HQ829120.1	Capnodiales	
	HM42	<i>Cladosporium cladosporioides</i>	+	+	+	+	+	+	+	+	100	HQ829120.2	Capnodiales	
30	HM43	<i>Scopulariopsis brevicaulis</i>	+	+	+	+	+	+	+	+	100	LN850790.1	Microascales	
	HM48	<i>Hormographiella aspergillata</i>	+	+	+	+	+	+	+	+	100	KP132299.1	Agaricales	
	HM49	<i>Trichosporon faecalis</i>	+	+	+	+	+	+	+	+	100	EF153624.1	Trichosporonales	
40	HM53	<i>Circinella sp.</i>	+	+	+	+	+	+	+	+	87	KF805760.1	Mucorales	
	HM54	<i>Circinella sp.</i>	+	+	+	+	+	+	+	+	87	KF805760.1	Mucorales	
70	HM56	<i>Scopulariopsis brevicaulis</i>	+	+	+	+	+	+	+	+	99	LN850786.1	Microascales	
	HM57	<i>Scopulariopsis brevicaulis</i>	+	+	+	+	+	+	+	+	98	KC311514.1	Microascales	
	HM59	<i>Dichotomomyces cejpaii</i>	+	+	+	+	+	+	+	+	100	KF706672.1	Eurotiales	
	HM60	<i>Purpureocillium lilacinum</i>	+	+	+	+	+	+	+	+	99	KC311490.1	Hypocreales	
	HM64	<i>Aspergillus tubingensis</i>	+	+	+	+	+	+	+	+	99	KP725001.1	Eurotiales	
	HT17	<i>Scopulariopsis brevicaulis</i>	+	+	+	+	+	+	+	+	98	KC311514.1	Microascales	
120	HM65	<i>Aspergillus versicolor</i>	+	+	+	+	+	+	+	+	100	FJ878627.1	Eurotiales	
	HM69	<i>Acremonium flavum</i>	+	+	+	+	+	+	+	+	99	HQ232037.1	Hypocreales	
	HM70	<i>Thielavia sp.</i>	+	+	+	+	+	+	+	+	99	GU055519.1	Sordariales	
	HM71	<i>Purpureocillium lilacinum</i>	+	+	+	+	+	+	+	+	100	KC157756.1	Hypocreales	
	HM74	<i>Bjerkandera sp.</i>	+	+	+	+	+	+	+	+	98	KT305937.1	Polyporales	
	HM78	<i>Dichotomomyces cejpaii</i>	+	+	+	+	+	+	+	+	97	KF706672.1	Eurotiales	
	HM81	<i>Aspergillus niger</i>	+	+	+	+	+	+	+	+	99	KU323591.1	Eurotiales	
	HM82	<i>Acremonium chrysogenum</i>	+	+	+	+	+	+	+	+	98	U57672.1	Hypocreales	

Figure 3. Heat map of the fungal isolates identified and the extracellular profile production for each isolate (blue: positive, yellow: negative). Identity (%): Percentage of nucleotide identity with the most similar GenBank sequence (Access. number (GenBank)) and order to which each sequence belongs.

Several fungi showed six activities or more, as is shown in Figure 3. Among them, strains identified as *Scopulariopsis brevicaulis* like HM03, HM43, HM56, HM57, HT17, and HM43 showed positive results for several enzymes. Except the last one, the remaining strains showed positive results for amyolytic, pectinolytic, hemicellulolytic, and cellulolytic activity. The HM03 strain showed peroxidase and polyphenol oxidase activity; three of them also showed proteolytic activity. These species were isolated in different stages of the process. The six strains belonging to *S. brevicaulis* corresponded to the bulking sample and to 30 and 70 days, *S. brevicaulis* HT17 isolated at 55 °C.

On the other hand, strains HM18 and HM48 were identified as *Hormographiella aspergilata* and showed a high number of enzyme activities, including proteases, amylases, ammonifying enzymes, as well as laccases, peroxidases, and polyphenol oxidase, tyrosinase, pectinases, hemicellulases, and cellulases. *Emericella nidulans* strain HM22 produced seven enzymatic activities; among them were amylolytic, ammonifying enzymes, tyrosinase, pectinase, hemicellulase, cellulase, laccase, and peroxidase. The strain *Aspergillus versicolor* HM30 showed a high rate of production of amylase, lipase, ammonifying enzymes, tyrosinase, pectinases, cellulose, peroxidase, and polyphenol oxidase. In the maturation stage, *Thielavia* sp. HM70 strain with the production of a wide set of lignocellulosic activity was remarkable. In particular, this strain showed nine enzymes, like protease, amylase, ammonifying enzymes and tyrosinase, pectinase, cellulase, laccase peroxidase, and polyphenol oxidase. *Bjerkandera* sp. HM70 showed positive results in six activities, mostly oxidoreductases like laccase, peroxidase, and polyphenol oxidase. Besides that, it showed hydrolytic activities, like cellulose, protease, and amylase.

In the case of bacteria, a total of 128 strains were tested for enzyme activity production. The strains with the highest number of enzymes in each sample were selected and finally, a total of 34 bacterial strains were molecularly identified. Accession numbers for the 16S rDNA sequences are MT305959-MT305987 for bacterial strains. In this sense, representatives of each composting sample were selected according to the conditions of isolation (mesophile or thermophile), morphotypes (colonies more frequent), and the number of enzymes produced. In Figure 4, the identification of selected bacteria and their extracellular enzymes activities are shown. The results obtained showed that 21 of these 34 bacteria strains belonged to Bacillales, which was the most representative order among the isolates identified and also with the highest number of enzyme activities (six or more). Otherwise, Pseudomonadales was represented by *P. formosensis*, with a wide number of enzyme activities. Within Actinomycetales, *Arrobacter creatinolyticus* BM25 and *Corynebacterium glutaminicum* BM85, between three and four activities were shown. Within *Lactobacillales*, two strains identified belonged to *Enterococcus faecium* (BM08 and BT08), with four and five activities, respectively.

B. pumilus, BM02, BM05, and BT02 (Figure 4) showed between six and seven enzymatic activities (proteolytic, amylolytic, pectinolytic, hemicellulolytic, peroxidases, and polyphenol oxidases). *B. pumilus* BT02 showed six positive results of enzyme activities like protease, amylase, pectinase, hemicellulase, peroxidase, and polyphenol oxidase. In the case of *B. pumilus* BM02, ammonifying activity was also detected. *B. stratosphericus* was identified in three strains, corresponding to BM20, BT09, and BM43, with *B. stratosphericus* BM20 being the strain with the highest number of enzymes showing positive results for pectinolytic, amylolytic, ammonifying, and hemicellulolytic activities, as well as for oxidative enzymes like peroxidase and polyphenol oxidase. Meanwhile, strain BT09 showed the same activities, except for ammonifying enzymes and hemicellulase; the strain BM43 showed the same activities as BM20, apart from amylase activity. *B. safensis* BM34 showed six positive results for pectinase, amylase, ammonifying enzymes, hemicellulase, peroxidase, and polyphenol oxidase. *P. formosensis* was the only species belonging to Pseudomonadales with a high rate of enzyme activities. In this case, both strains, BT47 and BT88, were isolates at 55 °C and showed positive results for six activities. *P. formosensis* BT47 showed positive results for amylolytic, ammonifying, pectinolytic activities, laccase, peroxidase, and polyphenol oxidase, and BT88 strain showed the same profile, but with proteolytic activity instead of amylolytic activity. *B. mojavensis* BM40 and BT64 showed positive results for amylase, hemicellulose, and cellulose activity.

Sample	Strain name	Phylogenetic identification	Hydrolytic				Lignocellulosic					Identity (%)	Access number (GenBank)	Order
			Peptidolytic	Amylolytic	P-solubilizing Lipase	Ammonifying	Tyrosinase	Pectinase	Hemicellulase	Cellulase	Laccase			
SS	BM02	<i>Bacillus pumilus</i>	■	■	■	■	■	■	■	■	■	99	KY085970.1	<i>Bacillales</i>
	BM05	<i>Bacillus pumilus</i>	■	■	■	■	■	■	■	■	■	99	JX860616.1	<i>Bacillales</i>
	BT01	<i>Bacillus paralicheniformis</i>	■	■	■	■	■	■	■	■	■	99	MG651169.1	<i>Bacillales</i>
B	BM08	<i>Enterococcus faecium</i>	■	■	■	■	■	■	■	■	■	100	CP025077.1	<i>Lactobacillales</i>
	BT02	<i>Bacillus pumilus</i>	■	■	■	■	■	■	■	■	■	100	JX860616.1	<i>Bacillales</i>
0	BM20	<i>Bacillus stratosphericus</i>	■	■	■	■	■	■	■	■	■	100	KY797998.1	<i>Bacillales</i>
	BT04	<i>Bacillus paralicheniformis</i>	■	■	■	■	■	■	■	■	■	100	MF928386.1	<i>Bacillales</i>
1	BM25	<i>Arthrobacter creatinolyticus</i>	■	■	■	■	■	■	■	■	■	99	JN644504.1	<i>Actinomycetales</i>
	BT08	<i>Enterococcus faecium</i>	■	■	■	■	■	■	■	■	■	99	CP025077.1	<i>Lactobacillales</i>
	BT09	<i>Bacillus stratosphericus</i>	■	■	■	■	■	■	■	■	■	99	KY797998.1	<i>Bacillales</i>
2	BT16	<i>Bacillus licheniformis</i>	■	■	■	■	■	■	■	■	■	100	MG546213.1	<i>Bacillales</i>
	BT18	<i>Bacillus paralicheniformis</i>	■	■	■	■	■	■	■	■	■	100	MG651169.1	<i>Bacillales</i>
5	BM34	<i>Bacillus safensis</i>	■	■	■	■	■	■	■	■	■	99	CP018100.1	<i>Bacillales</i>
	BM40	<i>Bacillus mojavensis</i>	■	■	■	■	■	■	■	■	■	100	MF040286.1	<i>Bacillales</i>
	BT23	<i>Bacillus beijingensis</i>	■	■	■	■	■	■	■	■	■	99	NR_117988.1	<i>Bacillales</i>
8	BM43	<i>Bacillus stratosphericus</i>	■	■	■	■	■	■	■	■	■	100	KY797998.1	<i>Bacillales</i>
	BM50	<i>Bacillus beijingensis</i>	■	■	■	■	■	■	■	■	■	99	NR_117988.1	<i>Bacillales</i>
	BT29	<i>Bacillus paralicheniformis</i>	■	■	■	■	■	■	■	■	■	99	MG651169.1	<i>Bacillales</i>
	BT30	<i>Bacillus paralicheniformis</i>	■	■	■	■	■	■	■	■	■	99	CP023168.1	<i>Bacillales</i>
14	BM60	<i>Virgibacillus halodenitrificans</i>	■	■	■	■	■	■	■	■	■	99	CP017962.1	<i>Bacillales</i>
	BM65	<i>Virgibacillus halodenitrificans</i>	■	■	■	■	■	■	■	■	■	99	CP017962.1	<i>Bacillales</i>
	BT37	<i>Bacillus licheniformis</i>	■	■	■	■	■	■	■	■	■	100	MG593200.1	<i>Bacillales</i>
30	BM70	<i>Serpens flexibilis</i>	■	■	■	■	■	■	■	■	■	98	KP973996.1	<i>Pseudomonadales</i>
	BM73	<i>Bacillus paralicheniformis</i>	■	■	■	■	■	■	■	■	■	100	CP023168.1	<i>Bacillales</i>
	BT47	<i>Pseudomonas formosensis</i>	■	■	■	■	■	■	■	■	■	100	NR_118141.1	<i>Pseudomonadales</i>
	BT57	<i>Pseudomonas formosensis</i>	■	■	■	■	■	■	■	■	■	100	NR_118141.1	<i>Pseudomonadales</i>
40	BM85	<i>Corynebacterium glutamicum</i>	■	■	■	■	■	■	■	■	■	99	CP013991.1	<i>Actinomycetales</i>
	BT64	<i>Bacillus mojavensis</i>	■	■	■	■	■	■	■	■	■	100	KY131800.1	<i>Bacillales</i>
70	BM96	<i>Pseudomonas formosensis</i>	■	■	■	■	■	■	■	■	■	100	NR_118141.1	<i>Pseudomonadales</i>
	BM100	<i>Psychrobacter pulmonis</i>	■	■	■	■	■	■	■	■	■	99	KU364016.1	<i>Pseudomonadales</i>
120	BM106	<i>Pseudomonas formosensis</i>	■	■	■	■	■	■	■	■	■	99	NR_118141.1	<i>Pseudomonadales</i>
	BM110	<i>Pseudomonas formosensis</i>	■	■	■	■	■	■	■	■	■	99	NR_118141.1	<i>Pseudomonadales</i>
	BT87	<i>Pseudomonas formosensis</i>	■	■	■	■	■	■	■	■	■	99	NR_118141.1	<i>Pseudomonadales</i>
	BT88	<i>Pseudomonas formosensis</i>	■	■	■	■	■	■	■	■	■	99	NR_118141.1	<i>Pseudomonadales</i>

Figure 4. Heat map of the bacterial isolates identified and the extracellular profile production for each isolate (blue: positive, yellow: negative). Identity (%): Percentage of nucleotide identity with the most similar GenBank sequence (Access. number (GenBank)) and order to which each sequence belongs.

4. Discussion

Composting is an oxidative process performed by microorganisms in which the temperature can reach 70 °C. This microbiota is strongly influenced by the microorganisms present in the starting material and the operational conditions, among other factors. In this study, a full-scale composting pile was built at industrial scale using a semipermeable cover connected with a ventilation system. Previous studies have observed that the temperature under these conditions can be higher than 80 °C at 8 days of the process [10,24] becoming an interesting ecosystem for fungi and bacteria able to resist high temperatures. In this study, a total of 105 fungal strains and 128 bacterial strains were initially isolated. However, in the case of fungi, only 40 could be maintained at laboratory conditions. The number of lost isolates could be explained because it is difficult to reproduce the same nutritional conditions of the pile in the laboratory with synthetic media.

All the strains were tested for hydrolytic and oxidative enzymes. The results showed a high percentage of isolates able to produce enzymes like polyphenol oxidase, peroxidase, amylase, and ammonifying enzymes, these being, the most frequent enzymes in the total of isolates.

For fungi, amylase was the most frequent enzyme, together with polyphenol oxidase and peroxidases, and, to a lesser extent, pectinase and protease. The number of isolates able to produce amylases was higher in the bulking agent (made of chips from the crushing and vegetable biomass from gardens) and in the samples at 0 and 1 day of composting. After that, the fungal strains isolated over 2, 5 and 8 days (corresponding with the highest temperature in the pile under cover stage) did not show high enzyme production. However, fungal strains isolated at 30, 70 and 120 days, showed a high number of lignocellulosic enzymes. This may be explained because these isolates were present also in the bulking agent and could be more active in mesophilic conditions. Isolates from the maturation stage showed a similar enzymatic profile compared to those isolated from the first phase of composting.

In the case of bacteria, polyphenol oxidase and peroxidase were the most predominant enzymes, confirming the role of these microorganisms in lignin degradation and their contribution to the humification process. It is well-known that ligninolytic enzymes, due to their non-specificity, are involved in the degradation of numerous recalcitrant aromatic compounds, such as hydrocarbons and emerging pollutants, dyes, and BTEX (benzene, toluene, ethylbenzene and xylene) [25,26]. These enzymes could also be involved in the removal of recalcitrant compounds frequently present in sewage sludge, such as pharmaceutical active compounds and polycyclic aromatic hydrocarbons, among others. Ammonifying activity was present in more than half of the bacterial isolates, which indicated the ability of the isolates in transformation of organic nitrogen to ammonium [27]. As well, few bacterial strains isolated between 0–5 days showed hemicellulolytic and amylolytic activities.

Regarding the distribution in the number of activities according to the composting time, our results suggest that some phases of composting can be more favourable in finding microorganisms with particular metabolic requirements and they can produce these enzymes in a different range of temperatures. A higher number of fungal isolates from the first stage and maturation stage were characterised by amylolytic, hemicellulolytic, and proteolytic activity, meanwhile, for bacteria, there were more isolates from the middle stage and maturation stage characterised by lignocellulosic enzymes.

Among the fungi, *Aspergillus* and *Scopulariopsis* had the highest number of strains and were isolated at different sampling times. The most frequent fungi isolated under semipermeable film were *Aspergillus*, *Circinella* (saprotrophic and nematophagous fungi), and *Talaromyces*. They have been described as fungi responsible for organic matter degradation in the thermophile phase due to their resistance to high temperatures (50–65 °C). These filamentous fungi belong to Hypocreales, Sordariales, and Microascales or orders with high representation in the mesophilic and thermophilic phases. A similar dynamic was described by Ryckeboer et al. [28], where members of *Aspergillus*, *Scopulariopsis*, and *Acremonium*, characterised this process. In this study, *Aspergillus versicolor* HM30 was isolated at the beginning of the process, a common inhabitant in all the environments. Previous studies have reported xylanase production by this species and its potential to bioaccumulate heavy metals and degrade dyes in water effluents [29,30]. All of these fungi, except *Scopulariopsis*, *Coprinopsis* (*Hormographiella*), and *Trichosporon*, have been identified in composting processes using pyrosequencing techniques [31]. However, *S. brevicaulis* has been isolated in the composting of lignocellulosic material, in the mesophilic and thermophilic phases [9]. This species has been recently described as degrader of polycyclic aromatic hydrocarbons (PAHs) like anthracene and described as a thermotolerant fungus [32,33]. In this pile, fungi such as *Hormographiella aspergilatta* and *Emericella nidulans* have not been previously described in the sewage sludge composting process and they showed a high number of enzymes. *Hormographiella aspergilatta*, an opportunist pathogen related to complications in patients undergoing treatments for lung diseases [34], could not be considered as a GRAS microorganism (Generally Recognised as Safe). However, its enzymes could be used from previous extraction or genetic engineering. As well, *Emericella nidulans* HM22 produced all the enzymes studied. The ability of this species to produce enzymes involved in cellulose degradation, like xylanase, has been reported previously [35]. This species has been isolated from soil and is considered a thermotolerant fungus, and could be regarded as a species with a potential biotechnological application and a good candidate for further studies.

It is interesting to note the presence in the maturation stage of *Thielavia* and *Bjerkandera* sp. *Thielavia* sp. HM70 showed positive results for nine enzymes, four of them corresponding to lignocellulosic enzymes. *Thielavia* is an ascomycete fungus that includes mesophilic and thermophilic species able to secrete up to five types of laccases [36,37]. *Bjerkandera* sp. HM70 was also identified in the maturation stage. This fungus is a white rot fungus, whose whole genome has been sequenced and it is characterised by the presence of a broad set of enzymatic activities, mainly peroxidases such as versatile peroxidase, manganese peroxidase, and inducible laccases in the presence of lignocellulosic material [38].

Concerning bacteria, *E. faecium* (strains BM08 and BT08) showed a remarkable activity. This bacteria has been widely isolated from water and soil samples and has been reported as an efficient degrader of aromatic compounds [39]. *Bacillus* was identified in several strains during the process. *B. pumilus*, BM02, BM05, and BT02 had a higher number of activities like proteolytic, amylolytic, pectinolytic, hemicellulolytic, peroxidase, and polyphenol oxidase. These results are in accordance with previous studies in which *B. licheniformis* and *B. pumilus* were described as polyphenol oxidase and peroxidase producers [40]. These strains showed a broad metabolic profile and have been previously characterised by their ability to produce peroxidases and proteases. The wide range of temperatures (present also in the thermotolerant range) and pH in which *Bacillus* can grow, increase their biotechnological applications [25,41]. As well, *B. safensis* BM34 showed a wide set of enzymes similar to *B. pumilus*. Recent studies have shown its potential to produce proteases, in particular alkaline proteases and their application in the detergent industry [42]. *B. mojavensis* showed only positive results for three enzymes but was isolated in a thermotolerant range. The effectiveness of *B. mojavensis* with the production of different enzymatic activities, like amylases and proteases in a thermophile range, has been previously reported [43]. Also, other studies have demonstrated their ability to produce lignocellulolytic enzymes like xylanase, which was not determined in our study [44,45].

Bacillus is a well-known genus present in composting processes [46–48], and this study showed the production of a broad numerous of extracellular enzymes. In addition, *Bacillus* species are known for their ability to tolerate high temperatures, in part, due to their ability to form spores. This genus has been widely described as a predominant genus in composting and is able to produce hemicellulolytic enzymes at 50 °C [49].

5. Conclusions

In this study we demonstrated that a real composting pile under semipermeable cover represents an interesting source of microorganisms with metabolic features of biotechnological interest. More studies are necessary to quantify, characterize, and purify these enzymes for further industrial applications, as well as to investigate the effectiveness of these enzymes in the degradation of recalcitrant compounds.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2076-3417/10/21/7763/s1>, Table S1. Summary table of methodology used to determine hydrolytic extracellular enzymes. Table S2. Summary table of methodology used to determine oxidative extracellular enzymes.

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