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Exploring the microbiota dynamics related to vegetable biomasses degradation and study of lignocellulose-degrading bacteria for industrial biotechnological application

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The aims of this study were to evaluate the microbial diversity of different lignocellulosic biomasses during degradation under natural conditions and to isolate, select, characterise new well-adapted bacterial strains to detect potentially improved enzyme-producing bacteria. The microbiota of biomass piles of *Arundo donax*, *Eucalyptus camaldulensis* and *Populus nigra* were evaluated by high-throughput sequencing. A highly complex bacterial community was found, composed of ubiquitous bacteria, with the highest representation by the *Actinobacteria*, *Proteobacteria*, *Bacteroidetes* and *Firmicutes* phyla. The abundances of the major and minor taxa retrieved during the process were determined by the selective pressure produced by the lignocellulosic plant species and degradation conditions. Moreover, cellulolytic bacteria were isolated using differential substrates and screened for cellulase, cellobiase, xylanase, pectinase and ligninase activities. Forty strains that showed multienzymatic activity were selected and identified. The highest endo-cellulase activity was seen in *Promicromonospora sukumoe* CE86 and *Isoptericola variabilis* CA84, which were able to degrade cellulose, cellobiose and xylan. Sixty-two percent of bacterial strains tested exhibited high extracellular *endo*-1,4-ß-glucanase activity in liquid media. These approaches show that the microbiota of lignocellulosic biomasses can be considered an important source of bacterial strains to upgrade the feasibility of lignocellulose conversion for the 'greener' technology of second-generation biofuels.

L ignocellulosic biomass includes dedicated energy crops, such as miscanthus, switchgrass, *Arundo donax*, *Populus nigra* and *Eucalyptus camaldulensis*. These plants can easily grow in farmland not suitable for food crops or in soils subjected to accelerated erosion, which affects most of the Mediterranean hilly croplands[1–3]. Vegetable biomasses represent an inexpensive alternative to fossil sources of fermentable sugars that can be utilised in several industrial applications, including second-generation biofuels and biopolymer production[4].

The enzymatic hydrolysis of plant carbohydrates has emerged as the most prominent eco-technology for the degradation of such biomasses. From the microbiological point of view, lignocellulosic biomass represents a complex ecosystem in which environmental conditions influence living organisms. In particular, geochemical (pH and salinity) and physical (temperature, pressure and radiation) factors can have a selective pressure on the biodiversity of microorganisms[5]. As a consequence, autochthonous microbial communities may prevail over other microorganisms because they possess enzymes that are able to degrade complex molecules such as cellulose and hemicellulose, forming the lignocellulosic biomasses that are the most abundant renewable energy source on Earth[6].

Generally, at an industrial level, the bioconversion of pretreated cellulose-based materials into fermentable sugars is performed by using a reaction mixture composed of multiple enzymes for complete hydrolysis. However, because the biorefining process is still economically unfeasible, novel biocatalysts from bacteria could

help overcome costly hurdles due to the operative steps of cooling, oxygen pumping, stirring and neutralisation, as well as the intrinsically high cost of hydrolytic enzyme production[7]. Different microorganisms producing hemicellulolytic enzymes that are potentially usable as new biocatalysts for hemicellulose hydrolysis have been isolated from different natural environments such as compost[8,9]. They belong to specific groups of microorganisms that are able to synthesise cellulase, xylanases and other biocatalysts necessary to allow a complete hydrolysis of the recalcitrant components of the lignocellulosic biomass[10]. Cellulolytic microorganisms can synthesise distinct enzymes such as endoglucanases, exoglucanases, including d-cellodextrinases, cellobiohydrolases and ß-glycosidase, which cooperate in cellulose degradation. Indeed, the hemicellulolytic microorganisms produce xylanases for degrading xylan into xylose that include endo-β-1,4-xylanases and β-xylosidases as well as auxiliary enzymes such as α-glucuronidases, α-arabinofuranosidases, acetylesterases and acetyl xylan esterases[11].

The use of culture-independent high-throughput sequencing can potentially reveal uncultivable microbiota and enables the study of the microbial ecology and taxonomic diversity at a high resolution. A thorough determination of the microbial diversity in biomass degradation can be fundamental to evaluating potential sources of novel enzymes and activities[12,13].

In the present work, the changes in the microbiota during the natural biodegradation of lignocellulosic biomasses of *A. donax*, *E. camaldulensis* and *P. nigra* were studied. In addition, new well-adapted bacterial strains from the three lignocellulose biomasses were isolated, identified and characterised. This study shows that the microbiota of lignocellulosic biomasses can be considered an important source of bacterial strains to upgrade the feasibility of lignocellulose conversion for the 'greener' technology of second-generation biofuels.

Results

Physicochemical measurement. The temperature values observed in the piles were approximately 24°C during the first 45 days. This value increased up to about 29°C after 135 days of biodegradation, before declining up to 25°C at the end of the experiment (180 days). The values of a_w ranged from to 0.91 to 0.99. The environmental temperature increased from April (20.5°C on the average) to August (34.2°C on the average) and declined in September (29.2°C).

Microbial Diversity of Lignocellulosic Biomasses by High-Throughput Sequencing. The microbiota of three different lignocellulosic biomasses (*A. donax*, *E. camaldulensis* and *P. nigra*) was characterised by partial 16S rRNA gene sequencing obtained from DNA directly extracted from environmental samples. A total of 238,450 number of reads were obtained by high-throughput sequencing. However, following the removal of short, ambiguous and/or low-quality pyrotag reads, the final data set consisted of 138,336 high-quality reads with an average sequence length of 456 bp. The alpha-diversity was determined by calculating the Shannon diversity index and the Chao1 richness index based on OTUs of 97% identity (Table 1). The results showed that the highest diversity indices were observed in the *E. camaldulensis* and *P. nigra* biomasses after 180 days of biodegradation in the underwood condition ($P < 0.05$) but were quite variable in the *A. donax* biomass (Table 1). Good's coverage indicated that more than 90% of the microbial diversity was described in most of the samples.

The relative abundances of bacterial taxa were examined at the level of phyla and class to determine whether there were any significant shifts in the composition of the bacterial communities according to the plant species, degradation conditions and sampling time.

In total, twenty-six different phyla were detected in the biomass samples, but only *Actinobacteria*, *Proteobacteria*, *Bacteroidetes* and *Firmicutes* were detected in all samples (Fig. 1). These phyla together

Table 1 | Number of sequences analysed, observed diversity and estimated sample coverage for 16S rRNA amplification from DNA extracted from the chipped lingo-cellulosic biomasses

Sample	No. reads	No. OTUs	Chao1	Shannon index[a]	Good's coverage (%)
At0	12,021	322	432.68	4.28[AB]	99.24
At1OF	6,235	477	682.92	6.59[DE]	97.45
At1UW	9,713	506	713.56	5.51[BC]	98.38
At2OF	4,158	592	955.73	7.09[DEF]	93.70
At2UW	2,564	286	443.81	6.09[C]	95.44
At3OF	1,699	404	618.70	7.33[F]	88.99
At3UW	5,451	441	561.42	6.36[CDE]	97.54
At4OF	5,160	620	1111.02	6.94[DEF]	94.46
At4UW	4,293	501	671.50	6.61[DE]	95.64
Et0	5,582	340	555.28	6.05[C]	97.76
Et1OF	5,271	322	485.11	3.92[A]	97.67
Et1UW	3,207	286	430.38	4.65[B]	96.35
Et2OF	3,983	453	656.84	6.65[DE]	95.81
Et2UW	1,069	228	405.60	6.52[DE]	89.5
Et3OF	4,520	470	671.11	6.52[DE]	96.00
Et3UW	7,091	623	978.51	6.46[CDE]	96.63
Et4OF	4,271	571	882.78	7.49[FG]	94.66
Et4UW	5,309	887	1367.25	8.37[GH]	93.60
Pt0	10,116	471	623.72	5.94[C]	98.65
Pt1OF	4,292	501	865.63	6.18[CD]	94.43
Pt1UW	10,117	845	1128.45	8.24[G]	97.68
Pt2OF	3,524	728	1097.07	7.89[FG]	91.06
Pt2UW	1,226	367	727.57	7.58[FG]	84.01
Pt3OF	5,031	743	1086.64	6.63[DE]	93.76
Pt3UW	2,188	583	1169.58	8.08[FG]	86.15
Pt4OF	2,576	654	975.19	7.82[FG]	88.94
Pt4UW	7,669	1,629	3122.43	9.48[H]	89.79

[a]Different letters after Shannon index values indicate significant differences ($P < 0.05$).
Abbreviations. A: *A. donax*; E: *E. camaldulensis*; P: *P. nigra*; T0: 0 days of degradation; t1: 45 days of degradation; t2: 90 days of degradation; t3: 135 days of degradation; t4: 180 days of degradation; OF: open field degradation condition; UW: underwood degradation condition.
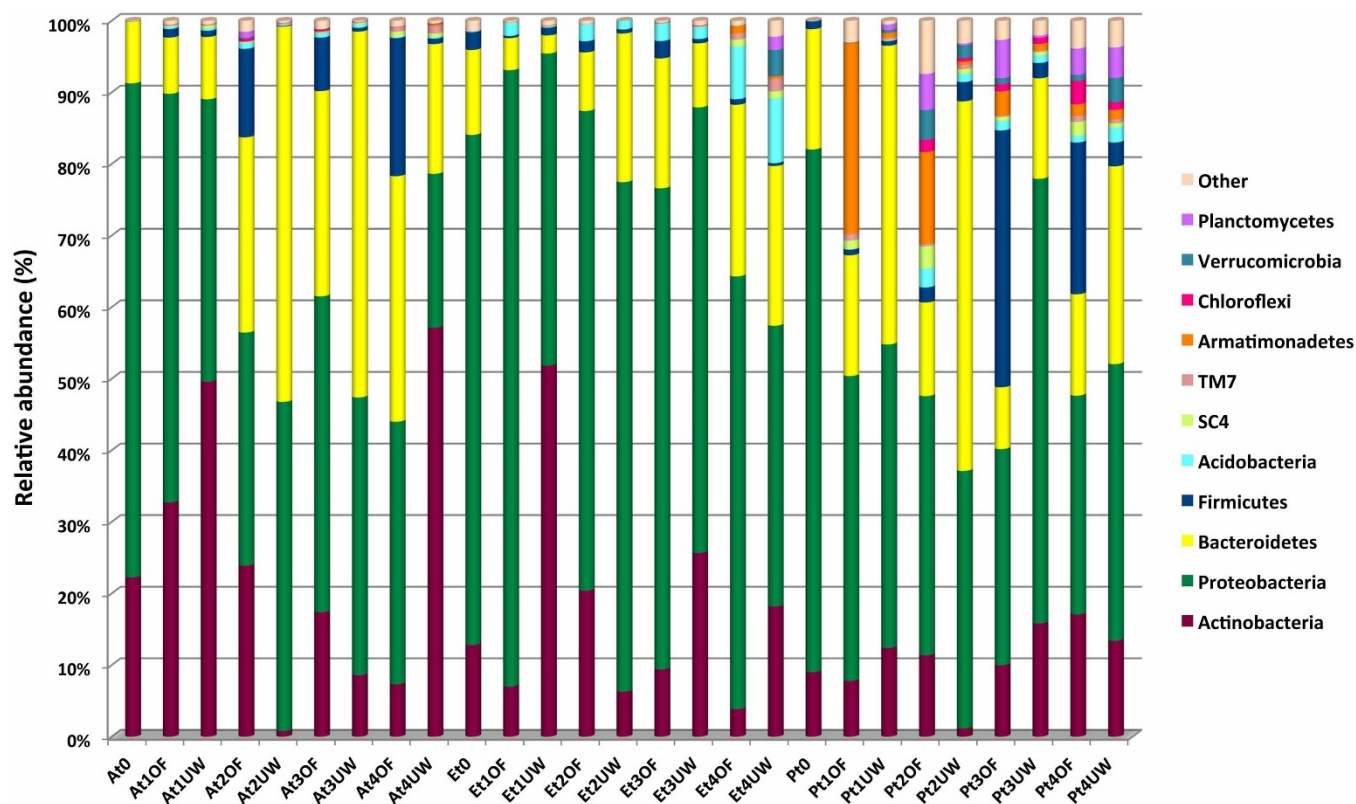
Figure 1 | Abundance of bacterial phyla in lignocellulosic biomasses during the biodegradation process. Only OTUs with an incidence >1% in at least two samples are shown. *Abbreviations.* A: *A. donax*; E: *E. camaldulensis*; P: *P. nigra*; T0: 0 days of degradation; t1: 45 days of degradation; t2: 90 days of degradation; t3: 135 days of degradation; t4: 180 days of degradation; OF: open field degradation condition; UW: underwood degradation condition.

accounted for approximately 98%, 99% and 91% of the total biodiversity in *A. donax*, *E. camaldulensis* and *P. nigra*, respectively.

The native composition of the microbial community in the *A. donax* biomass was strongly dominated by *Proteobacteria* (69.03%), followed by *Actinobacteria* (22.23%) and *Bacteroidetes* (8.61%). During the biodegradation process, the microbial composition remained the same in terms of diversity but varied in terms of abundance. This pattern was primarily observed in the piles processed under the open field condition (Fig. 1). Interestingly, in this environment, an inverse correlation was observed between *Actinobacteria* and *Bacteroidetes*. In particular, *Actinobacteria* decreased from 32.70% after 45 days of degradation under the open field condition to 7.31% after 180 days ($P < 0.05$), whereas *Bacteroidetes* increased from 7.84% after 45 days to 34.30% after 180 days ($P < 0.05$). *Firmicutes* was very low at the initial time (0.02%) and remained roughly constant in the underwood pile (0.77% after 180 days of biodegradation). By contrast, in the open field pile, the relative abundance of this taxon gradually increased from 1.20% after 45 days of biodegradation to 19.28% after 180 days ($P < 0.05$). Minor phyla included *Acidobacteria* and candidate phyla SC4 and TM7 were recovered in all *A. donax* biomass samples, although at an incidence ≤ 1% (Fig. 1).

As in the *A. donax* biomass, *Proteobacteria* was the taxa that heavily dominated the native microbial community in *E. camaldulensis* and *P. nigra* (71.26% and 73.01%, respectively) ($P < 0.05$), remaining high during all degradation processes, followed by *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Acidobacteria* (Fig. 1). In the *E. camaldulensis* pile, *Acidobacteria* increased to 7.35% and 9.02% after 180 days of degradation under the open field and underwood conditions, respectively, whereas a slight variation was detected in the *P. nigra* samples. By contrast, the abundance of *Firmicutes* increased in the *P. nigra* pile after 135 (35.88%, $P < 0.05$) and 180 days (21.20%, $P < 0.05$) of degradation under the open field condition and was relatively stable in the *E. camaldulensis* pile (Fig. 1). Other taxa, including

Armatimonadetes, *Chloroflexi*, *Planctomycetes*, *Verrucomicrobia*, SC4 and TM7, were also detected. Interestingly, *Armatimonadetes* increased in the *P. nigra* pile after 45 days under the open field condition (26.70%, $P < 0.05$) and then gradually decreased during the degradation process to 1.63% (Fig. 1).

The microbial diversity was also analysed at a deeper taxonomic level. The identification of OTUs at the class level is reported in the heatmap shown in Fig. 2. *Actinobacteria*, *γ-Proteobacteria*, *β-Proteobacteria*, *α-Proteobacteria*, *Acidobacteria*, *Sphingobacteria*, *Flavobacteria* and *Bacilli* were recovered in all samples, with the exception of *Acidobacteria*, which was not detected in the *A. donax* biomass at the initial time. The abundance of α-*Proteobacteria* was high and exhibited the same variations during the biodegradation experiment in all three lignocellulosic biomasses. Interestingly, *γ-Proteobacteria* was the dominant taxa at the beginning of the experiment, with an abundance of 58.32%, 42.64% and 67.48% in the *A. donax*, *E. camaldulensis* and *P. nigra* samples, respectively; however, a dramatic reduction was recorded in all plant biomasses after 180 days (1.07%, 8.12% and 11.49%, respectively) (Fig. 2). A steady reduction in class *Actinobacteria* during degradation of the *A. donax* biomass under the open field condition was observed. By contrast, *Bacilli* showed an opposite trend, increasing from 0.02% at time zero to 19.26% after 180 days. Under the underwood condition, the middle phase of the biodegradation process was primarily dominated by *Sphingobacteria*, which showed an abundance of approximately 50%. Ever-increasing *Sphingobacteria* and *β-Proteobacteria* taxa abundance was recorded in the *E. camaldulensis* pile during the degradation process under the open field experiment. *Acidobacteria* showed a similar trend under both the open field and underwood conditions. The *P. nigra* biomass was characterised by an increase in the incidence of *Bacilli* and *β-Proteobacteria* during the biodegradation process under the open field conditions (Fig. 2). Other taxa were present at a very low incidence and with a great variability in the different
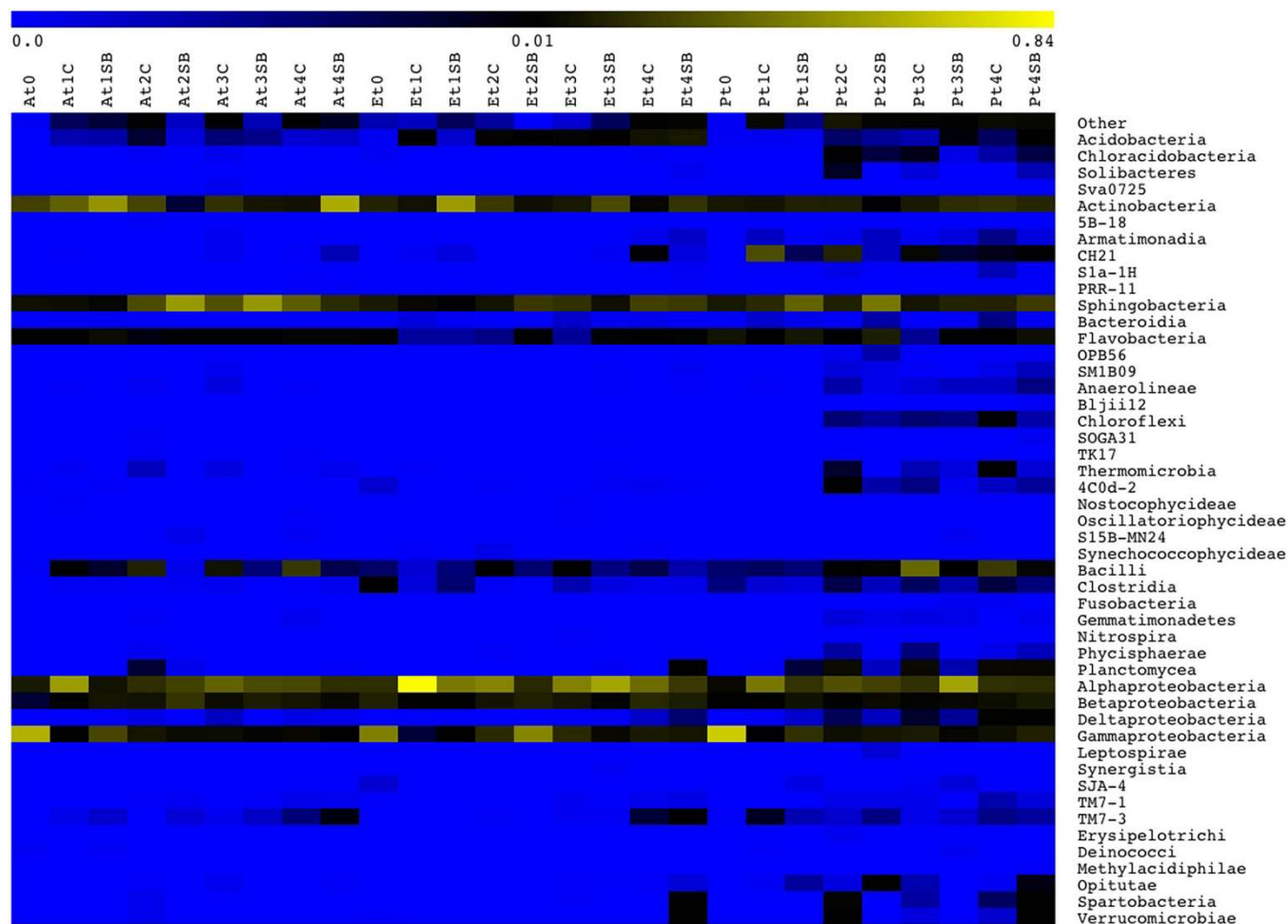
Figure 2 | Distribution of bacterial classes in lignocellulosic biomasses during the biodegradation process. Colour scale indicates the relative abundance of each OTU within the samples. *Abbreviations.* A: *A. donax*; E: *E. camaldulensis*; P: *P. nigra*; T0: 0 days of degradation; t1: 45 days of degradation; t2: 90 days of degradation; t3: 135 days of degradation; t4: 180 days of degradation; OF: open field degradation condition; UW: underwood degradation condition.

samples. However, as shown in Fig. 2, the highest bacterial diversity was recorded in the *P. nigra* biomass. An interesting finding was the high incidence of the uncultured bacterium CH21 (26.45%) in the *P. nigra* pile during the first phase (after 45 days) and its constant decrease during the other phases of the degradation process in the open field. *Chloroflexi* was detected only in the *P. nigra* biomass and from 90 days to 180 days of biodegradation, showing an abundance ranging from 0.33% to 1.55%.

However, the PCoA of the weighted UniFrac community distances showed a marked difference between the native microbiota of the lignocellulosic biomasses and the microbiota in the analysed samples during the biodegradation process. The three samples of the chipped biomasses of *A. donax*, *E. camaldulensis* and *P. nigra* at harvest time clustered separately (Fig. 3). However, the microbial diversity seemed to be influenced by a correlation between the degradation condition and the biomass type. In fact, no difference was observed among the samples during the biodegradation process under the underwood condition (Fig. 3 panel a). By contrast, under the open field condition, *P. nigra* samples at different time points clustered separately from the other biomasses (Fig. 3, panel b). A similar trend was observed in the *A. donax* biomass, except for the samples collected after 45 days of degradation (Fig. 3, panel b). Moreover, the statistical ADONIS test showed that the composition of bacterial community in the different piles during biodegradation process was significantly influenced by lignocellulosic plant species ($P < 0.001$), by the degradation conditions ($P < 0.05$) and by sam-

pling time ($P < 0.01$). This difference increased combining the three factors. In fact, the analysis performed with ADONIS found significant difference combining *lignocellulosic plant species* x *sampling time* ($P < 0.001$), *lignocellulosic plant species* x *degradation condition* ($P < 0.001$) and *degradation condition* x *sampling time* ($P < 0.01$).

Multienzymatic Screening and Identification of Isolated Cellulolytic Bacterial Strains. A culture-dependent approach enables the isolation of putative cellulolytic strains. A total of 366 aerobic endo- and exo-cellulolytic bacteria were isolated from samples of the *A. donax*, *E. camaldulensis*, and *P. nigra* biomasses. In particular, more than one hundred isolates were obtained from each pile, although the *P. nigra* biomass produced the highest number (127 isolates). In total, 95.9% of the isolates that were obtained from Avicel agar plates, exhibited exo-cellulase activity, whereas isolates with endo-cellulase activity on the CMC agar plates were detected at values less than 50%. To establish the number of putative multifunctional-degrading bacteria, all isolates were assayed for different enzymatic activities using the methods described below. Forty aerobic endo- and exo-cellulolytic strains that showed higher I_{CMC} values (> 10) were chosen for further characterisation. The polyphasic approach of identification resulted in bacterial isolates with different shapes, dimensions and, in some cases, spore presence (data not shown) and great biodiversity, as twenty genera and twenty-seven different species were found (Table 2). *Curtobacterium* spp. and *Bacillus* spp. were the most representative genera with the species *Curtobacterium flaccumfaciens* and
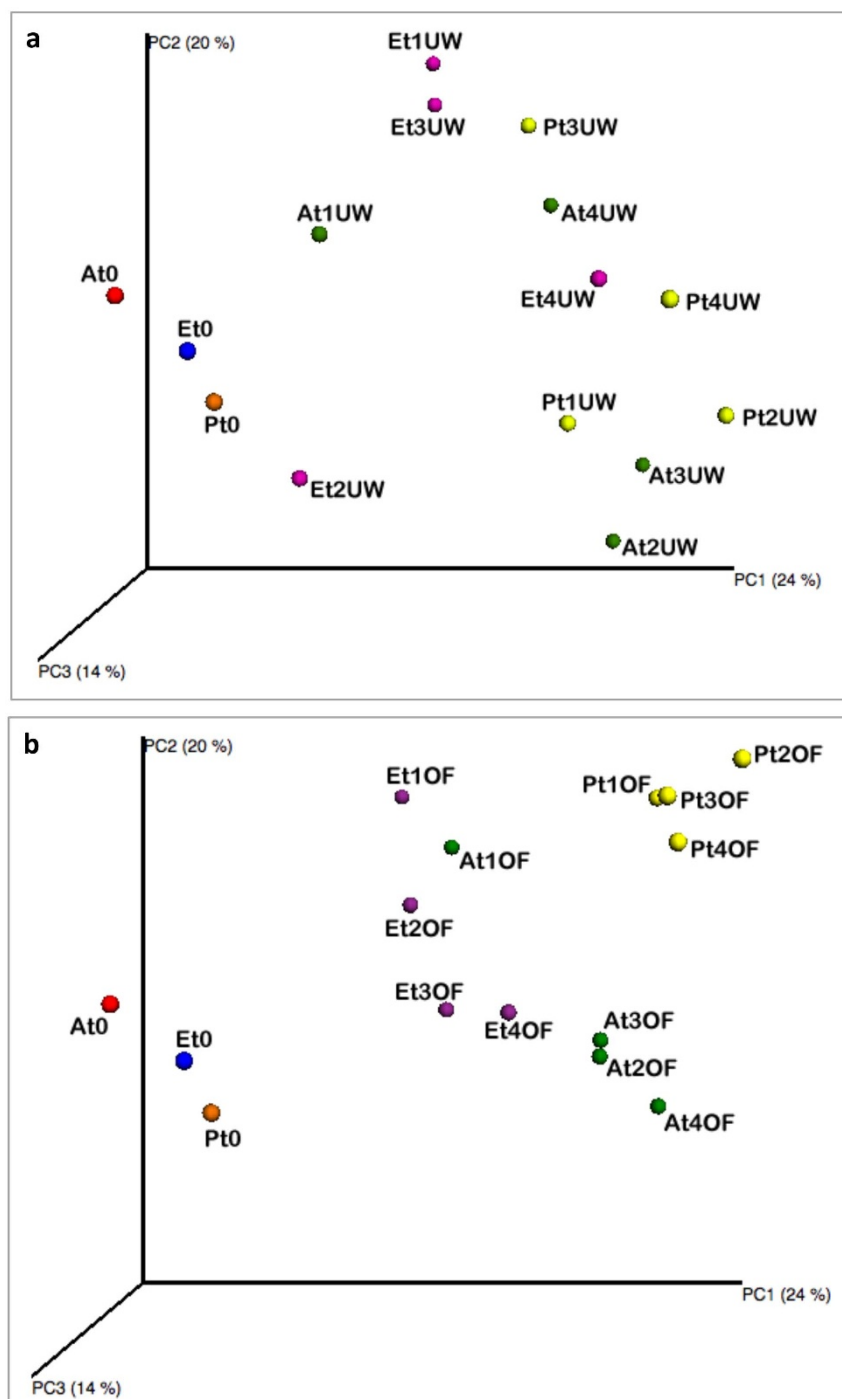
Figure 3 | Principal Coordinates Analysis of weighted UniFrac distances for 16S rRNA gene sequence data of lignocellulosic biomasses during the biodegradation process under the underwood (panel a) and open field (panel b) conditions. *Colour label.* red: *A. donax* at harvest time; blue: *E. camaldulensis* at harvest time; orange: *P. nigra* at harvest time; green: *A. donax*; violet: *E. camaldulensis*; yellow: *P. nigra*. *Abbreviations.* A: *A. donax*; E: *E. camaldulensis*; P: *P. nigra*; T0: 0 days of degradation; t1: 45 days of degradation; t2: 90 days of degradation; t3: 135 days of degradation; t4: 180 days of degradation; OF: open field degradation condition; UW: underwood degradation condition.

Curtobacterium citreum as well as *Bacillus amyloliquefaciens*, *Bacillus licheniformis* and *Bacillus subtilis*, respectively. The bacterial strains showed activities for three to six different enzymes. All strains showed endo- and exo-cellulolytic activities and, at same time, expressed cellobiase, xylanase and pectinase (Table 2). The highest endo-cellulase activity was observed in the CE86 strain, identified as *Promicromonospora sukumoe* ($I_{CMC} = 34$, $P < 0.05$), in *Isoptericola variabilis* CA84b ($I_{CMC} = 32$, $P < 0.05$) and *Staphylococcus warneri* CE83 ($I_{CMC} = 32$, $P < 0.05$). The first two strains were also able to

degrade exo-cellulose, cellobiose and xylan. Moreover, *Isoptericola variabilis* CA84b produced the highest pectinase activity ($I_{PEC} = 22$, $P < 0.05$). *Curtobacterium citreum* CE711 and *Bacillus amyloliquefaciens* CA81 represented the most versatile strains because they possessed six different enzymatic activities. Thirty-two bacterial strains showed, in addition to endo- and exo-cellulases, cellobiase and xylanase activities that were missed in only 10% of the screened strains (*Staphylococcus warneri* CE83, *Lysobacter enzymogenes* CE710, *Lysobacter gummosus* CP72, *Mycobacterium frederiksbergense*

Table 2 | Identification and enzymatic activities of bacterial strains isolated from different lignocellulosic biomasses

Strain	Source	C[a]	A[b]	CE[c]	X[c]	P[a]	AZ[c]	AB[c]	L[c]	AD[c]	Identification (% identity)	Accession Number
CE86	E. camaldulensis	34 ± 1.0[N]	+	++	+	0.0 ± 0.0[A]	-	-	-	-	Promicromonospora sukumoe (99%)	KF057947
CA84b	A. donax	32 ± 0.0[N]	+	++	+	22 ± 0.5[G]	-	-	-	-	Isoptericola variabilis (98%)	KF057948
CE83	E. camaldulensis	32 ± 1.0[N]	+	+	-	0.0 ± 0.0[A]	-	-	-	-	Staphylococcus warneri (100%)	KF057949
CP72	P. nigra	28 ± 1.0[M]	+	-	++	0.0 ± 0.0[A]	-	-	-	-	Lysobacter gummosus (99%)	KF040972
CP710b	P. nigra	28 ± 0.5[M]	+	-	+	12 ± 0.5[E]	-	-	-	-	Mycobacterium frederiksbergense (99%)	KF057950
CA817	A. donax	27 ± 0.5[M]	+	+	+	2 ± 0.2[B]	-	-	-	-	Cellulosimicrobium cellulans (99%)	KF040973
CA812	A. donax	27 ± 1.0[M]	+	+++	+++	0.0 ± 0.0[A]	-	-	-	-	Bacillus amyloliquefaciens (99%)	KF040974
SBP79	P. nigra	26 ± 0.5[LM]	+	++	+	12 ± 0.0[E]	-	-	-	-	Promicromonospora citrea (99%)	KF057951
SBA88	A. donax	26 ± 0.3[LM]	+	+	+	0.0 ± 0.0[A]	-	-	-	-	Microbacterium lacus (99%)	KF057952
SBE74	E. camaldulensis	24 ± 1.0[IL]	+	±	+	0.0 ± 0.0[A]	-	-	-	-	Enterobacter aerogenes/Kluyvera cryocrescens (98%)	KF057960
CE77	E. camaldulensis	22 ± 1.0[HI]	+	-	-	0.0 ± 0.0[A]	++	-	-	-	Novosphingobium resinovorum (100%)	KF040976
CP81	P. nigra	22 ± 0.5[HI]	+	+	+	3 ± 0.0[C]	-	-	-	-	Xanthomonas campestris (100%)	KF040971
CP77	P. nigra	22 ± 0.9[HI]	+	+	+	4 ± 0.0[D]	-	-	-	-	Xanthomonas orizae (98%)	KF040975
CE75b	E. camaldulensis	22 ± 0.2[HI]	+	++	++	0.0 ± 0.0[A]	-	-	-	-	Pediococcus acidilactici (99%)	KF057953
CP77b	P. nigra	22 ± 0.5[HI]	+	++	±	0.0 ± 0.0[A]	-	-	-	-	Curtobacterium flaccumfaciens (99%)	KF057954
SBA76	A. donax	22 ± 1.0[HI]	+	+	+	0.0 ± 0.0[A]	-	-	-	-	Pantoea ananatis (99%)	KF057955
SBP71	P. nigra	22 ± 1.0[HI]	+	+	+	0.0 ± 0.0[A]	-	-	-	-	Schumannella luteola (97%)	KF057956
CP78b	P. nigra	22 ± 0.0[HI]	+	±	+	12 ± 0.0[E]	-	-	-	-	Mycobacterium frederiksbergense (99%)	KF057957
CA816	A. donax	21 ± 0.5[H]	+	+++	-	20 ± 1.0[F]	-	-	-	-	Bacillus subtilis subsp. subtilis (99%)	KF040977
CA81	A. donax	20 ± 1.0[GH]	+	+++	+++	0.0 ± 0.0[A]	+	+	-	-	Bacillus amyloliquefaciens (99%)	KF040978
CA82	A. donax	20 ± 0.0[GH]	+	+++	++	4 ± 0.2[D]	-	-	-	-	Bacillus licheniformis (99%)	KF040979
CE84	E. camaldulensis	20 ± 0.5[GH]	+	+	+++	0.0 ± 0.0[A]	++	-	-	-	Novosphingobium resinovorum (99%)	KF040980
CA81b	A. donax	20 ± 1.0[GH]	+	+++	++	4 ± 0.1[D]	-	-	-	-	Bacillus licheniformis (99%)	KF040981
CE73b	E. camaldulensis	20 ± 0.9[GH]	+	++	+	0.0 ± 0.0[A]	-	-	-	-	Pediococcus acidilactici (99%)	KF057958
SBP73	P. nigra	20 ± 0.0[GH]	+	-	±	0.0 ± 0.0[A]	-	-	-	-	Aurantimonas altamirensis (99%)	KF057959
CE85	E. camaldulensis	18 ± 1.0[FG]	+	++	++	2 ± 0.0[B]	-	-	-	-	Isoptericola variabilis (98%)	KF040982
CE710	E. camaldulensis	18 ± 0.5[FG]	+	-	++	2 ± 0.1[B]	-	++	-	-	Lysobacter enzymogenes (99%)	KF040983
CP81b	P. nigra	18 ± 1.0[FG]	+	++	++	0.0 ± 0.0[A]	-	-	-	-	Promicromonospora sukumoe (99%)	KF040984
CA83	A. donax	17 ± 1.0[EF]	+	++	+++	2 ± 0.1[B]	-	-	-	-	Curtobacterium flaccumfaciens (99%)	KF040985
CA77	A. donax	16 ± 0.5[DEF]	+	++	+	2 ± 0.0[B]	-	-	-	-	Sphingobacterium multivorum (99%)	KF040986
CP75	E. camaldulensis	16 ± 1.0[DEF]	+	++	+	2 ± 0.1[B]	-	-	-	-	Curtobacterium flaccumfaciens (99%)	KF040987
CP73	E. camaldulensis	16 ± 0.5[DEF]	+	+	-	0.0 ± 0.0[A]	-	-	-	-	Promicromonospora citrea (99%)	KF040988
CA83b	A. donax	15 ± 0.3[CDE]	+	++	++	0.0 ± 0.0[A]	-	-	-	-	Curtobacterium flaccumfaciens (99%)	KF040989
CA84	A. donax	14 ± 0.5[BCD]	+	+++	+	0.0 ± 0.0[A]	-	-	-	-	Curtobacterium citreum (99%)	KF040990
CP713	P. nigra	14 ± 0.3[BCD]	+	+++	++	20 ± 0.3[F]	-	-	-	-	Cellulomonas flavigena (98%)	KF040991
CA818	A. donax	13 ± 1.0[BC]	+	++	+	2 ± 0.0[B]	-	-	-	-	Microbacterium testaceum (99%)	KF040992
CE78	E. camaldulensis	12 ± 0.0[AB]	+	++	+	2 ± 0.3[B]	-	-	-	-	Curtobacterium citreum (99%)	KF040993
CP710	P. nigra	12 ± 0.5[AB]	+	+	+	0.0 ± 0.0[A]	-	-	-	-	Labedella gwakjiensis (99%)	KF040994
CE711	E. camaldulensis	10 ± 0.9[A]	+	++	+	2 ± 0.0[B]	+	-	-	-	Curtobacterium citreum (99%)	KF040995
CP78	P. nigra	10 ± 0.5[A]	+	+++	+	0.0 ± 0.0[A]	-	-	-	-	Raoultella terrigena (99%)	KF040996

Enzymatic activities: C = endo-cellulase; A = eso-cellulase; CE = cellobiase; X = xylanase; P = pectinase; AZ = peroxidase; AB = laccase; L = ligninase with guaiacol and lignin alkali; AD = ligninase with guaiacol and Arundo donax; [a]CMC or I_{FEC} index, values represent the means ± SD of three replicates. Different letters after values indicate significant differences ($P<0.05$); [b]growth; [c] − negative; + low intensity; ++ middle intensity; +++ high intensity.
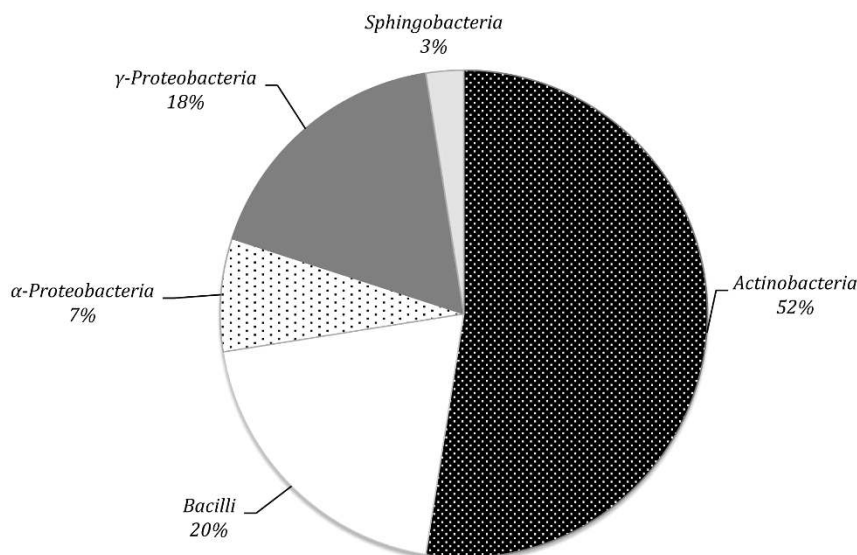
Figure 4 | Percentage composition of different phyla of eso- and endo-cellulolytic bacteria isolated from lignocellulosic biomasses on the basis of 16S rRNA gene sequence similarity.

CP710b, *Promicromonospora citrea* CE73, *Novosphingobium resinovorum* CE77, *Bacillus subtilis* subsp. *subtilis* CA816 and *Aurantimonas altamirensis* SBP73). All the strains belonging to the different *Bacillus* spp. and *Curtobacterium* spp. had the highest cellobiase activities. In particular, the genus *Curtobacterium,* represented by the species *Curtobacterium citreum* and *Curtobacterium flaccumfaciens,* was able to grow at least on four/five different substrates and, in one case, also showed peroxidase activity. In total, 87.5% of the bacterial strains produced xylanase (Table 2). Similar behaviour was observed for pectinase activity production as only 15% of the strains possessed an I_{PEC} from 12 to 22. Laccase and peroxidase activities were detected in five strains belonging to the species *Curtobacterium citreum* CE711, *Novosphingobium resinovorum* CE77 and CE84, *Lysobacter enzymogenes* CE710 and *Bacillus amyloliquefaciens* CA81. Lignin hydrolysis was not detected in any of the strains tested.

Phylogenetic Analysis of Selected Cellulolytic Bacteria. 16S rRNA gene sequences of the forty cellulolytic bacteria identified as described below were grouped by phylogenetic analysis into five different clusters, generating a *consensus* tree. The clusters were *Actinobacteria* (n=21; 52.5%), *Bacilli* (n=8; 20.0%), *α-Proteobacteria* (n=3; 7.5%), *γ-Proteobacteria* (n=7; 17.5%), *Sphingobacteria* (n=1; 4%) (Fig. 4). A phylogenetic tree was generated from the distance data using the Neighbour-Joining method with the Maximum Composite Likelihood model in a MEGA4 Program (Fig. 5). The nucleotide sequences of related type strains of different genera were included in the data set. High bootstrap values were observed and indicated significant branching points in the phylogenetic tree. Strains representative of the dominant class of *Actinobacteria* were placed in a cluster with bootstrap values higher than 52% and could be differentiated into nine subclusters of different genera. The bacterial strains primarily belonged to *Curtobacterium* (33.3%) and *Promicromonospora* (19.0%), whereas other genera (*Schumannella, Labedella, Cellulomonas, Cellulosimicrobium, Microbacterium, Isoptericola,* and *Mycobacterium*) were represented by one or two strains. The strains *Curtobacterium citreum* CE711, *Curtobacterium citreum* CA84 and *Curtobacterium citreum* CE78 showed a sequence similarity of 100%. Representatives of the *Bacilli* class were placed in three clusters of the genera *Bacillus* (62.5%), *Staphylococcus* (12.5%) and *Pediococcus* (25.0%). The strains belonging to *Bacillus licheniformis* CA82 and CA81b showed a similarity level of 100%, as did *Bacillus amyloliqui-*

quefaciens CA812 and CA81 and *Pediococcus acidilactici* CE75b and CE73b (Fig. 5). The *α-Proteobacteria* strains were placed in two different genera, in which two strains of *Novosphingobium resivorum* (CE77 and CE84) exhibited a sequence similarity of 100% with 100% of the bootstrap analysis. The other strain, belonging to *Aurantimonas altamirensis* (SBP73), formed a phylogenetically distinct cluster with a low 16S rRNA gene sequence similarity (60%) (Fig. 5). The representative strains of the *γ-Proteobacteria* cluster belonged to the genera *Xanthomonas* and *Lysobacter* and included the species *Xanthomonas campestris, Xanthomonas oryzae, Lysobacter gummosus,* and *Lysobacter enzymogenes.* The other genera, identified as the *Enterobacter aerogenes/Klebsiella cryocrescens* group, *Raoultella* and *Pantoea,* were represented by one strain. The strain *Sphingobacterium multivorum* CA77 formed a phylogenetically distinct cluster with the type strain of the respective species with 100% of the bootstrap analysis (Fig. 5).

Screening of Cellulolytic Microorganisms in Liquid Medium. Sixteen bacterial strains were selected based on at least one of these characteristics: higher halo dimension on CMC agar, multienzymatic activities, high pectinase activity or high peroxidase activity, and no pathogenicity for humans; these strains were submitted to a quantitative CMCase assay. All the analysed strains achieving the maximum AZO-CMCase level of production between the 8th and 26th hours, corresponding to the exponential and stationary phases of growth, respectively (data not shown). In Table 3, the values of the maximum AZO-CMCase activity measured for each strain and the corresponding time of production are reported. *Schumannella luteola* SBP71 showed the highest AZO-CMCase activity, at 0.33 ± 0.09 U mL^{-1}, although no significant differences were found with other strains. In particular, high activity levels ranging from 0.20 ± 0.06 to 0.32 ± 0.08 U mL^{-1} were also found for *Pediococcus acidilactici* CE75b, *Curtobacterium citreum* CA84, *Curtobacterium flaccumfaciens* CA83, *Cellulomonas flavigena* CP713 and *Pantoea ananatis* SBA76a (Table 3). *Mycobacterium frederiksbergense* CP78b, *Curtobacterium flaccumfaciens* CP77b and *Promicromonospora citrea* SBP79 (with a maximum value of 0.18 ± 0.02 U mL^{-1} and 0.16 ± 0.02 U mL^{-1}) exhibited less AZO-CMCase activity than the microorganisms mentioned above but more than *Cellulosimicrobium cellulans* CA817 (0.15 ± 0.01 U mL^{-1}) and *Isoptericola variabilis* CA84b (0.13 ± 0.01 U mL^{-1}). All other bacterial strains tested showed values lower than 0.10 U mL^{-1}.
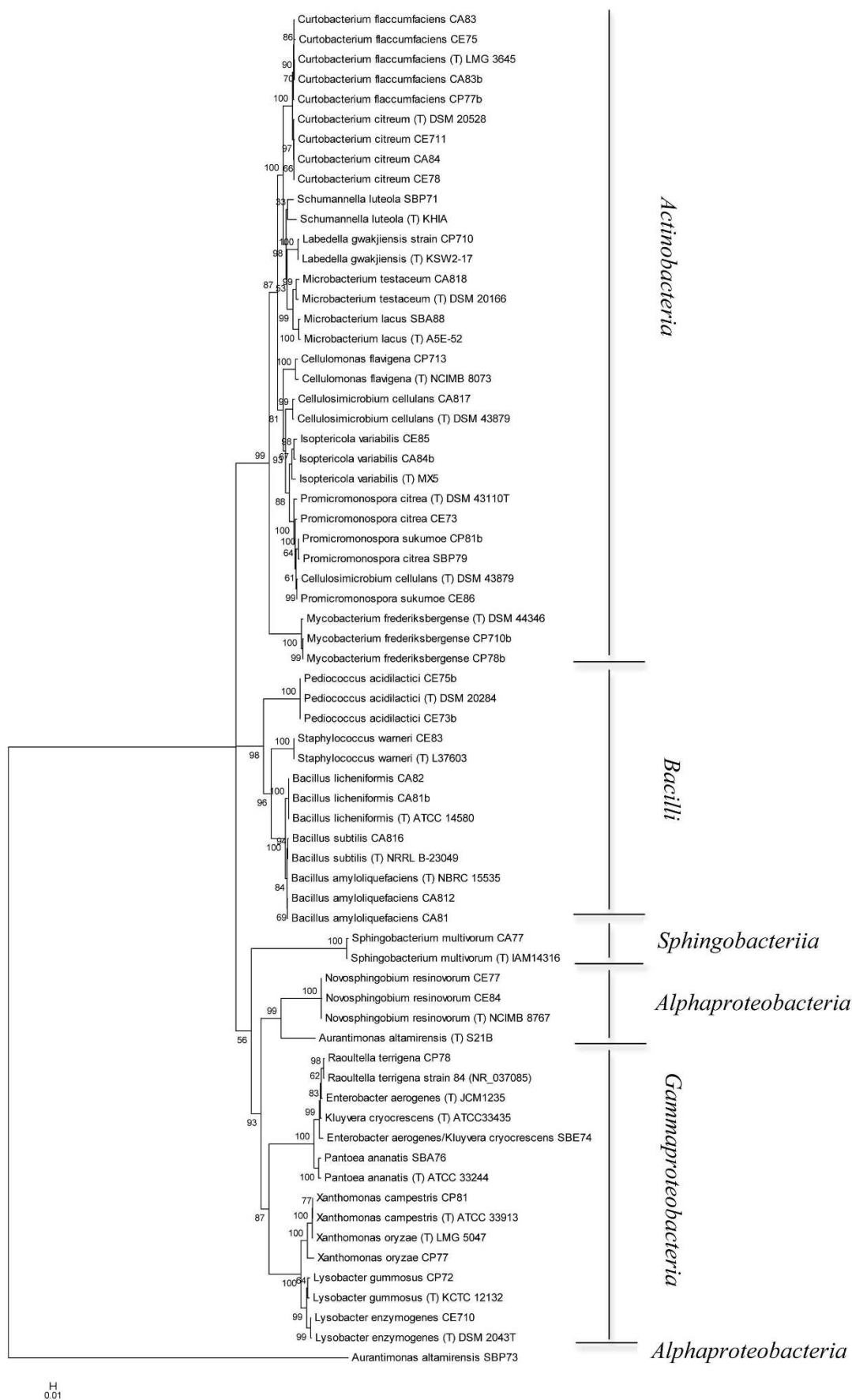
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Figure 5 | Neighbour-Joining tree based on the comparison of 16S rRNA gene sequences showing the relationships among cellulolytic strains. Bootstrap values (expressed as percentages of 1,000 replications) greater than 50% are given at the nodes. Strains marked with "(T)" represent type strains. The *scale bar* estimates the number of substitutions per site.

Table 3 | Maximum value of Azo-CMCase activity measured for each strain and the corresponding time of production

Bacterial strains	Maximum value of AZO-CMCase activity[a] (U mL^{-1})	Time (h)
Schumannella luteola SBP71	0.33 ± 0.09[F]	12
Pediococcus acidilactici CE75b	0.32 ± 0.10[EF]	12
Curtobacterium citreum CA84	0.29 ± 0.01[D-F]	17
Curtobacterium flaccumfaciens CA83	0.24 ± 0.03[C-F]	15
Cellulomonas flavigena CP713	0.22 ± 0.10[B-F]	20
Pantoea ananatis SBA76a	0.20 ± 0.06[A-F]	8
Mycobacterium frederiksbergense CP78b	0.18 ± 0.02[A-E]	26
Curtobacterium flaccumfaciens CP77b	0.16 ± 0.02[A-D]	20
Promicromonospora citrea SBP79	0.16 ± 0.02[A-D]	12
Cellulosimicrobium cellulans CA817	0.15 ± 0.01[A-D]	20
Isoptericola variabilis CA84b	0.13 ± 0.01[A-C]	12
Bacillus subtilis subsp. *subtilis* CA816	0.09 ± 0.00[AB]	12
Microbacterium testaceum CA818	0.09 ± 0.01[AB]	17
Curtobacterium flaccumfaciens CA83b	0.08 ± 0.03[AB]	12
Promicromonospora sukumoe CE86	0.07 ± 0.02[A]	14
Bacillus licheniformis CA82	0.07 ± 0.00[A]	15

[a]The values represent the means ± SD of three replicates of three independent experiments. Different letters after the values indicate significant differences ($P < 0.05$).

Discussion

In recent years, the competitive production of alternative renewable biofuels has stimulated research into new bacteria as a source of highly active and specific cellulases. They exhibit several advantages such as a fast growth rate, production of enzymes that are often more effective catalysts due to less feedback inhibition, and secretion of a complete multi-enzyme system for an efficient conversion of ligno-celluloses into fermentable sugars[11,14]. In this context, particular attention must be given to exploring the biodiversity of natural niches so that cellulase-producing bacteria can be isolated and characterised. For these reasons, in this work, the microbial diversity of natural ecosystems, represented by lignocellulosic biomasses of *A. donax*, *E. camaldulensis* and *P. nigra*, was evaluated by culture-independent and culture-dependent approaches.

A highly complex bacterial community was found, in which the most frequently occurring bacteria were those belonging to the *Actinobacteria*, *Proteobacteria*, *Bacteroidetes* and *Firmicutes* phyla. *Proteobacteria* was the most abundant taxa recovered in the *E. camaldulensis* and *P. nigra* piles, followed by *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Acidobacteria*. These taxa are related to microorganisms previously characterised as biomass degraders. The biodiversity of the microbial community in our study corresponded well with a previous study in which *Proteobacteria*, *Firmicutes* and *Bacteroidetes*, along with members of the class *Proteobacteria*, comprised 83% of the microbial richness and heavily dominated switchgrass-adapted communities[15]. Moreover, bacterial species belonging to *Proteobacteria* and *Acidobacteria*, together with *Firmicutes* (*Clostridium* and *Bacillus* genera) and followed by *Bacteroidetes*, *Chlamydiae/Verrucomicrobia* and *Actinobacteria* (mainly Streptomyces), are known as the major plant biomass-degrading microbes in peat swamp forests[16]. During the natural biodegradation process of plant substrates, the indigenous bacterial community would initially have grown by utilising the more accessible cellulose and hemicellulose and only later would use the more resilient lignin component[17]. With regard to lignin decomposition, *Actinobacteria*, *Firmicutes* and *Acidobacteria* are the major taxa involved in this process[18]. *Acidobacteria* was recovered in the late stage of our experiment and its abundance increased during the biodegradation process, especially in the *E. camaldulensis* piles. *Firmicutes* showed a similar trend in the *A. donax* and *P. nigra* biomasses, with the relative abundance of this taxon gradually increasing in the piles processed under the open field condition. Wu and He[19] reported that *Firmicutes* could be the main microbes for lignin depolymerisation since a dominance of this phylum was recovered in enriched microbial consortia using a medium with lig-

nin. Moreover, this phylum is common in natural processes such as rice straw compost[20] and decaying wood[21], suggesting its importance in the degradation of lignocellulolytic materials.

Analysing the microbial diversity more deeply, *Actinobacteria*, *γ-Proteobacteria*, *β-Proteobacteria*, *α-Proteobacteria*, *Acidobacteria*, *Sphingobacteria*, *Flavobacteria*, *Bacilli* and *Acidobacteria* were recovered in all samples. In particular, *Actinobacteria*, *α-Proteobacteria*, *Sphingobacteria* and *β-Proteobacteria*, all potent plant polysaccharide-degrading microbes that play an important role in plant biomass degradation in the tropical peat swamp forest ecosystem[16], were the most abundant taxa during the biodegradation process in all lignocellulosic piles.

Different bacteria belonging to the *Actinobacteria* class are involved in complex glycoside degradation such as chitin and cellulose and are fundamental in lignin and polyphenol degradation[22]. Martins and co-workers[23] reported that biomass degradation in the composting process, including the deconstruction of recalcitrant lignocellulose, is fully performed by bacterial enzymes, most likely by members of the *Clostridiales* and *Actinomycetales* orders. *β-Proteobacteria* and *α-Proteobacteria* were also recovered in our study. According to Castillo et al.[22], the dominance of members of the phylum *Proteobacteria* such as *γ-Proteobacteria* is observed only at the beginning of the biodegradation process, and its strong reduction during the experiment could be due to its involvement in lignocellulosic waste declining during the early stages of the process. In the open field experiment, *Bacilli* increased in both the *A. donax* and *P. nigra* biomasses. Members belonging to this taxon are known to have specific genes encoding enzymes involved in cellulose and hemicellulose degradation[24,25].

The constant increase in the relative abundance of *Sphingobacteria* and *Acidobacteria* recorded in the *E. camaldulensis* pile during the degradation process in the open field experiment suggests that these species play a role in the decomposition of lignocellulosic material. Kanokratana et al.[26], using complementary shotgun pyrosequencing, identified different genes encoding glycosyl hydrolases targeting cellulose and hemicellulose degradation in a bagasse pile, most of which were found in orders *Clostridiales*, *Bacteroidales*, *Sphingobacteriales* and *Cytophagales*. Moreover, the *Acidobactria* taxon is able to a use a diversity of carbon sources, from simple sugars to complex plant biomass substrates[26].

Another adapted-lignocellulosic taxon was *β-Proteobacteria*, which increased in the *E. camaldulensis* and *P. nigra* piles. In recent work, Stursova et al.[27] identified *β-Proteobacteria*, *Bacteroidetes* and *Acidobacteria* as the primary cellulose decomposers in forest litter.

An interesting finding was the high incidence of the uncultured bacterium CH21 in the *P. nigra* pile during the first phase of the

degradation process in the open field. CH21 is a member of the phylum *Armatimonadetes*, formerly called candidate division OP10[28]. The phylum *Armatimonadetes* is very poorly studied and its phylogeny is still poorly defined[29]. Members of this phylum are detected in different ecosystems and they are phylogenetically different. This phylum includes species with a wide variety of metabolic potentials[30]. Wang and co-workers[31] reported that their prevalence in plant-fed anaerobic bioreactors indicates a role in degradation of plant material. In our research, CH21 is the only recovered member of this phylum. Since it's relative abundance increased in the *Populus nigra* pile after 45 days and decreased during the other phases of the degradation process in the open field, it is possible its involving in the biodegradation of this specific lignocellulosic biomass during the first phase of the process.

The differing trends observed in this study in terms of taxa abundance during the biodegradation process and between the vegetable species used demonstrated a local selective pressure in the lignocellulosic ecosystems.

The culture-dependent methodology used here, which was based on a functional approach of detection and isolation to find new lignocellulose-degrading bacterial strains, provides us with key insight. Special attention to the methodology was required to determine the optimal culture and assay conditions. A comparison with another study[11] revealed that differential substrates containing CMC and Avicel are effective for the enumeration and isolation of putative colonies of cellulolytic microorganisms[32]. According to Soares et al.[6], exo- and endo-cellulolytic bacterial isolates are found at different frequencies, and the number of microorganisms that were able to grow on Avicel as the sole carbon source was high. The cellulolytic strains isolated from the biomasses showed multienzymatic activities useful to perform the hydrolysis of a complex substrate such as lignocellulose, an important initial step in many technological applications[33] that require the action of different specific enzymes. All forty bacterial strains submitted to the multienzymatic screening showed both endo- and exo-glucanase activities, confirming that these enzymes act synergistically during the saccharification of celluloses. These observations were reinforced by the fact that many of these bacterial strains also possessed β-cellobiase as well as cellulolytic activity. Xylanase activity was also commonly observed, which is unsurprising because a close correlation between cellulase and xylanase activities has been demonstrated and is due to their coexpression in the same operon.

The Neighbour-Joining phylogenetic method generated a consensus tree that grouped all 16S rRNA gene sequences of the isolated strains into five different clusters at the class level. Culture-dependent data showed similar predominant bacterial classes detected by high-throughput sequencing in the lignocellulosic biomasses, with an abundance of *Actinobacteria*, *Bacilli*, α-*Proteobacteria*, γ-*Proteobacteria* and *Sphingobacteria*. Moreover, the bacteria isolated are dominant players since these microbial strains were isolated from a high serial decimal dilutions (10^{-6}–10^{-7}). According to phylogenetic research on cellulose-decomposing bacteria isolated from soil carried out by Ulrich et al.[34], *Actinobacteria* are the most prevalent bacterial group based on 16S rRNA gene sequences. This cluster included the species *Curtobacterium citreum*, which is able to use up to six different lignocellulose components and that is phylogenetically related to *Microbacterium testaceum* and *Microbacterium lacus*. In particular, previous studies reported the production of enzymes involved in cellulose and xylan degradation by *Microbacterium* species[35]. The *Actinobacteria* cluster included other genera involved in lignocellulosic biomass degradation, such as *Cellulomonas*. Akasaka et al.[36] reported that more than 60% of isolates from rice plant residues was closely related to *Cellulomonas* and involved in their degradation. Our study revealed the production of cellobiase and pectinase activities in *Actinobacteria* members, such as *Cellulosimicrobium cellulans*, *Isoptericola variabilis*, *Promicromonospora sukumoe* and *Promicromonospora citrea*, which belong to the suborder *Micrococcineae*. Many of the representatives of the *Promicrosporaceae* and *Corinebacteriaceae* families can degrade polysaccharides such as cellulose and xylan[37,38]. The *Bacilli* cluster was primarily represented by the *Pediococcus* and *Bacillus* genera on the basis of 16S rRNA gene sequence analysis. Interesting, Zhao and co-workers[39] reported the use of a strain of *Pediococcus acidilactici* with high tolerance to temperature and a lignocellulose-derived inhibitor in simultaneous saccharification and fermentation (SSF) for high lignocellulosic lactic acid production. The strains CA812, CA81, CA816, CA82 and CA81b, isolated from the *A. donax* biomass and phylogenetically correlated to the *Bacillus* genus, showed high endo-cellulolytic and multi-enzymatic activities. The capacity of *Bacillus* strains to produce large quantities of extracellular enzymes has placed them among the most important industrial enzyme producers isolated from compost, soil and several other natural habitats[40]. In particular, *Bacillus amyloliquefaciens*, *Bacillus subtilis* and *Bacillus licheniformis*, isolated from soil and compost, are able to hydrolyse cellulosic waste-material[8] by both cellulolytic activities[41] and multi-enzyme complexes[42]. The selected bacterial strains could have cross-specificity facilitated by specific or non-specific active sites and also distinct catalytic domains binding to different substrates[33]. *Xanthomonas campestris*, known as a phytopathogenic bacterium, and *Lysobacter gummosus* and *Lysobacter enzymogenes*, potent biocontrol agents that release cellulolytic enzymes such as glucanase[43], were identified in the phylogenetic group of γ-*Proteobacteria*. Other strains of this class, such as *Enterobacter* sp. and *Pantoea* sp., although are known as insect-associated bacteria that are able to produce bioactive compounds and digestive enzymes that are responsible for lignocellulose degradation[44], they are less attractive for possible biotechnological application since human disease has been reported to be caused by these bacteria as well as *Raoultella terrigena* and *Sphingobacterium multivorum*.

The two strains CE77 and CE84, included in the α-*Proteobacteria* cluster and characterised for their peroxidase activity, were closely related to the species *Novosphingobium resinovorum*, previously isolated from soil and studied for its capacity to degrade oil resins[45]. Interestingly, the strain *Aurantimonas altamirensis* SBP73 showed multienzymatic activity. To our knowledge, this study is the first to report the multienzymatic activity of this specie.

The bacterial strains screened in liquid medium showed enzymatic activity levels similar to the values reported in previous studies[46]. For example, Ekperigin[47] reported that the maximum activity of the cellulose-degrading enzyme determined for CMC in the culture supernatant of *Branhamella* spp. was 0.34 U mL^{-1}. Even though comparing cellulase production across studies is difficult because there are too many differences in the production of extracellular enzymes (e.g., media composition, fermentation conditions and raw materials), the results demonstrated that the bacterial strains isolated and characterised in this study could represent a very interesting biological source for the conversion of lignocellulose carbohydrates into products of commercial significance. Moreover, their biotechnological performance could be enhanced by modifying the biotechnological parameters of the fermentation process such as the temperature[33].

In conclusion, in this work, pyrosequencing-based technology increased our knowledge of lignocellulosic-adapted microbiota and the microbial dynamic during the degradation of three different biomasses under natural conditions. The dominant taxa found during the biodegradation process were members of classes *Actinobacteria*, γ-*Proteobacteria*, α-*Proteobacteria* and *Sphingobacteria*. However, the abundance of the major and minor taxa retrieved during the process was determined by selective pressure employed by the lignocellulosic plant species and the degradation conditions.

In addition, new multifunctional degrading bacteria have been selected and identified that are potential producers of multiple enzymes that have synergistic actions on cellulose and hemicellulose.

This step is fundamental for biotechnological applications of interest to industry because they constitute a microbial source of new multi-functional enzymes that can increase the efficiency of the hydrolysis of lignocellulosic biomass into fermentable sugars for biofuel eco-technology.

Methods

Lignocellulosic Biomasses and Sampling. Chipped wood from *A. donax*, *E. camaldulensis* and *P. nigra* was processed for biodegradation under natural conditions for 180 days. The chipped wood was used to form two piles of approximately 30 kg (length 0.85 m, width 0.85 m and height 0.70 m) for each plant species; on March 22, 2012, the piles were placed under two different environmental conditions in order to increase microbial biodiversity: open field conditions, in which the biomass piles were established on agronomic soil without any coverage shady trees, in the experimental station of Department of Agriculture (Naples, Italy; 40°48′50.1″N, 14°20′48.2″E); the underwood conditions performed reproducing the natural environment that usually arise in a forest since the piles were placed under oak trees in the woodland at the Department of Agriculture (Naples, Italy; 40°48′47.8″N, 14°20′50.4″E). Samples of 0.5 kg were collected from the external part (right and left side of the pile) and the internal central part of the biomass immediately after preparation (T0) and at 45, 90, 135 and 180 days (T1, T2, T3 and T4, respectively) of biodegradation. During the process, temperature (°C) was measured by using specific temperature sensors (VWR International PBI, Milan, Italy) placed directly in the core of the piles at a depth of 30–40 cm. The water activity (a_w, water readily available for microbial metabolic activities) was also monitored using a HygroPalm23-AW (Rotronic AG, Basserdorf, Germany).

Analysis of the Microbiota by High-Throughput Sequencing of the 16S rRNA Gene. Total DNA recovery from microorganisms adherent to the plant biomass was performed as previously described[48]. The bacterial diversity was evaluated by pyrosequencing using the primers Gray28F (5′-TTTGATCNTGGCTCAG-3′) and Gray519r (5′-GTNTTACNGCGGCKGCTG-3′) spanning the V1-V3 region of the 16S rRNA of *E. coli*[49]. 454-adaptors were included in the forward primer followed by a 10-bp sample-specific Multiplex Identifier (MID). The PCR mixture (50-μl total volume) included 20 ng of target DNA, 1 x *Taq* DNA polymerase buffer (Invitrogen, Milano, Italy), 2.5 mM $MgCl_2$, 0.5 mM of each dNTP, 0.4 μM of each primer and 2.5 U of *Taq* DNA polymerase (Invitrogen). PCR amplification was performed as previously reported[50]. After agarose gel electrophoresis, PCR products were purified twice by Agencourt AMPure kit (Beckman Coulter, Milano, Italy) and quantified using the QuantiFluor™ (Promega, Milano, Italy), and an equimolar pool was obtained prior to further processing. The amplicon pool was used for pyrosequencing on a GS Junior platform (454 Life Sciences, Roche, Italy) according to the manufacturer's instructions using Titanium chemistry.

Bioinformatics and Data Analysis. The pyrosequencing data were filtered using the following quality check parameters using the QIIME software package[51]: a minimum sequence length of 200 bp, maximum homopolymer number of 5, minimum quality score of 25, and maximum number of ambiguous bases and primer mismatches of 0. The QIIME software was also used to denoise, split sequences into the proper samples, and pick operational taxonomy units (OTUs) at 97% sequence identity using UCLUST[52]. The representative sequences were submitted to the RDPII classifier[53] to obtain a taxonomy assignment using the Greengenes 16S rRNA gene database[54].

Alpha diversity was evaluated by rarefaction curves, Good's coverage, Chao1 richness[55] and Shannon diversity indices[56]. To test for significant differences in alpha diversity, QIIME's compare_alpha_diversity.py script was used to run nonparametric two-sample *t*-tests. The default number of Monte Carlo permutations (999) was used to calculate *P*-values in the nonparametric *t*-tests, and a significance threshold of $P < 0.05$ was used. Statistical tests for calculating the influence of sample variables on microbial ecology were applied using QIIME's group_significance.py (ANOVA) and compare_categories.py (ADONIS) scripts. The OTU taxonomy tables generated by QIIME were used to produce heatmaps using the software TMeV v 4.8[57]. Beta diversity was also evaluated by UniFrac[58], and principal coordinate analysis (PCoA) was generated by QIIME. Unweighted UniFrac distances were also calculated, but we only report weighted distances here because sample clustering was more informative.

Isolation of Cellulolytic Functional Groups in the Lignocellulosic Biomasses. Different samples were collected from the chipped vegetable biomass piles of *A. donax*, *E. camaldulensis* and *P. nigra* for the isolation of aerobic cellulolytic bacteria. Serial dilutions (0.1 mL) of the samples were spread on the surface of plates containing basal medium (1 g L^{-1} [NH_4]NO_3, 1 g L^{-1} yeast extract, 50 mL L^{-1} standard salt solution, 1 mL L^{-1} trace element solution and 15 g L^{-1} agar bacteriological, pH 7.0) with 5 g L^{-1}Avicel (Sigma-Aldrich, Milan, Italy) or 5 g L^{-1} carboxymethylcellulose (CMC) with 0.1% Congo red (Sigma-Aldrich) for exo-cellulolytic or endo-cellulolytic differential isolation, respectively. After 4 days under aerobic conditions at 28°C, the endo-cellulolytic colonies were differentially visualised by a clear halo on CMC. All colonies grown on the substrate Avicel as only carbon source were considered as exo-cellulolytic bacteria. Morphologically different exo- and endo-cellulolytic colonies were isolated, purified again on the same isolation media and stored at 4°C until their characterisation.

Screening for Functional Activities on Solid Media. The endo-cellulolytic activity of all isolates was measured on CMC by a semi-quantitative agar spot method. After adjusting the turbidity of the bacterial suspensions (0.5 of McFarland Turbidity Standard corresponding to approximately 1.5×10^8 CFU mL^{-1}), the cells were spotted on CMC agar medium in triplicate. After a 4-day incubation at 28°C, the plates were stained with 0.1% Congo red solution for 30 min followed by washing with 5 M NaCl[8,14]. Cellulase activities on screening media were recorded as the "Indices of Relative Enzyme Activity, I_{CMC}= diameter of clearing or halo zone/colony diameter"[11]. The exo-cellulase activity was similarly estimated in a semi-quantitative way by inoculating all the isolates by spotting on the Avicel agar and observing the development of bacterial colonies after incubation at 28°C for 10 days.

All isolates were also assayed for cellobiase, xylanase, pectinase and ligninase activities. In particular, cellobiase activity was assessed with cellobiose agar (10 g L^{-1} bacteriological peptone, 15 g L^{-1} agar bacteriological [Oxoid, Milan, Italy], 7.5 g L^{-1} cellobiose and 0.06 g L^{-1} bromothymol blue [Sigma-Aldrich]). After incubation at 28°C for 3 days, the cellobiase activity was detected by observing yellow haloes around the colonies. For xylanase detection, the method described by Ko et al.[59] was applied, adding 0.05% Remazol brilliant blue-R and a 0.5% solution of sonicated (FALC Instruments, HK3300) xylan (Sigma-Aldrich) to the LB Agar medium (Oxoid). After 3–5 days of incubation at 28°C, the hydrolysis of xylan was detected by observing a clear zone around the colonies. Pectinase activity was measured following the method described by Pepe et al.[60] and the results were recorded as the "Indices of Relative Enzyme Activity, (I_{PEC}) = diameter of clearing or halo zone/colony diameter".

Ligninase activity was detected by using the CGA culture medium described by Okino et al.[61], modified by replacing the sugarcane bagasse lignin with vegetable biomass powder from *A. donax* or 2 g L^{-1} lignin alkali (Sigma-Aldrich). A reddish-brown halo around a colony indicates the successful oxidation of guaiacol by ligni-nolytic enzymes. Laccase activity was determined for all the isolated strains by growth on agar medium composed of liquid basal medium (LBM) supplemented with 0.1% 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid [ABTS], Sigma-Aldrich) as described by Toutella et al.[62]. After 10 days of incubation at 25°C in darkness, a green colour in the medium indicated the presence of laccase activity. Azure-B (Sigma-Aldrich) agar was used to evaluate the production of lignin peroxidase and man-ganese-dependent peroxidase by the formation of a clear halo around a colony after 10–30 days of incubation at 25°C in darkness[63].

Identification and Phylogenetic Analysis of Cellulolytic Strains. Bacterial isolates were identified by molecular method (16S rRNA gene sequencing). Total genomic DNA was extracted and purified by InstaGene™ Matrix (Bio-Rad Laboratories, Hercules, CA, USA) according to the supplier's recommendations. Approximately 50 ng of DNA was used as the template for PCR assays. Synthetic oligonucleotide primers fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rD1 (5′-AAGGAGGTGATCCAGCC-3′) were used to amplify the 16S rRNA gene. The PCR mixture was prepared as reported by Alfonzo et al.[64]. The PCR conditions were performed as described by Pepe et al.[65]. The PCR products, after visualisation by agarose (1.5% wt/vol) gel electrophoresis at 100 V for 1 h, were purified by using a QIAquick gel extraction kit (Qiagen S.p.A., Milan, Italy) and sequenced. The DNA sequences were analysed as previously reported[61] and compared with the GenBank nucleotide data library using Blast software at the National Centre of Biotechnology Information website (http://www.ncbi.nlm.nih.gov/Blast.cgi).

Multiple nucleotide alignments of nearly full-length 16S rRNA sequences of iso-lated strains and type strains within each of the defined species were performed using the Clustal W program[66] from MEGA version 4.0[67]. The nucleotide sequences of the type strains were retrieved from the Ribosomal Database Project (RDP - http://rdp.cme.msu.edu/). The phylogenetic tree was inferred by using the Neighbour-Joining method with the Maximum Composite Likelihood model in MEGA4 program with bootstrap values based on 1,000 replications.

Screening of Cellulolytic Activity in Liquid Media. The bacterial strains were pre-inoculated by dissolving a single colony in 3 mL of liquid medium containing 0.5% CMC, 0.7% yeast extract, 4 g L^{-1} KH_2PO_4, 4 g L^{-1} Na_2HPO_4, 0.2 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.001 g L^{-1} $CaCl_2 \cdot 2H_2O$ and 0.004 g L^{-1} $FeSO_4 \cdot 7H_2O$. After overnight incubation at 37°C, a volume of the broth culture corresponding to 0.1 O.D. was used to inoculate 100-mL plugged Erlenmeyer flasks, each containing 20 mL of the same medium. During incubation at 37°C on a rotary shaker at 225 rpm, samples of the liquid culture were withdrawn and used to measure the optical density (O.D.$_{600nm}$) and the extracellular *endo*-1,4-ß-glucanase activity by AZO-CMCase assay (Megazyme, Ireland), following the supplier's instructions. The analytical determinations correspond to the mean value of three replicates.

Accession Numbers. The 16S rRNA gene sequences obtained were deposited in the GenBank nucleotide database under accession numbers from KF040971 to KF040996 and from KF057947 to KF057960 (http://www.ncbi.nlm.nih.gov/Blast.cgi).

The pyrosequencing data are available in the Sequence Read Archive database of the National Center of Biotechnology Information (PRJNA248067).

Statistical Analyses. One-way ANOVA followed by Tukey's HSD *post hoc* for pair-wise comparison of means (at $P < 0.05$) was used to assess the difference in the

enzymatic activities of isolated strains such as I_{CMC}, I_{PEC} and Azo-CMCase. Statistical analyses were performed using SPSS 13.0 statistical software package (SPSS Inc., Cary, NC, USA).

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Author contributions

V.V. wrote the main manuscript text and, in particular, microbial diversity of lignocellulosic biomasses by High-Throughput Sequencing and Phylogenetic analysis of selected cellulolytic bacteria. She prepared Figure 1, 2, 3, 4, 5 and table 1. A.Aliberti and A.R. wrote the isolation and identification of cellulolytic bacterial strains by culture-dependent approach and selection by multienzymatic screening. They prepared Table 2. V.F., S.G. and A.Amore wrote the screening of cellulolytic microorganisms in liquid medium and prepared Table 3. D.E. wrote the microbial diversity of lignocellulosic biomasses by High-Throughput Sequencing. M.F. wrote lignocellulosic biomasses and sampling. O.P. is the corresponding author. All authors reviewed the manuscript.

Additional information

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