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Exploring the Natural Fungal Biodiversity of Tropical and Temperate Forests toward Improvement of Biomass Conversion

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In this study, natural fungal diversity in wood-decaying species was explored for biomass deconstruction. In 2007 and 2008, fungal isolates were collected in temperate forests mainly from metropolitan France and in tropical forests mainly from French Guiana. We recovered and identified 74 monomorph cultures using morphological and molecular identification tools. Following production of fungal secretomes under inductive conditions, we evaluated the capacity of these fungal strains to potentiate a commercial *Trichoderma reesei* cellulase cocktail for the release of soluble sugars from biomass. The secretome of 19 isolates led to an improvement in biomass conversion of at least 23%. Of the isolates, the *Trametes gibbosa* BRFM 952 (Banque de Ressources Fongiques de Marseille) secretome performed best, with 60% improved conversion, a feature that was not universal to the *Trametes* and related genera. Enzymatic characterization of the *T. gibbosa* BRFM 952 secretome revealed an unexpected high activity on crystalline cellulose, higher than that of the *T. reesei* cellulase cocktail. This report highlights the interest in a systematic high-throughput assessment of collected fungal biodiversity to improve the enzymatic conversion of lignocellulosic biomass. It enabled the unbiased identification of new fungal strains issued from biodiversity with high biotechnological potential.

The magnitude of fungal diversity is estimated to be 1.5 million species, but only 5% of species have been described. This estimate was calculated from the ratio of fungal species to vascular plant species for various ecologically defined groups of fungi in well-studied regions (14, 15). In the light of data from site inventories, the fungus/plant ratio ranges from 5:1 to 33:1. Available evidence also indicates that fungal diversity in the tropics is richer than that in temperate regions (2, 15). The true scale of fungal diversity is still controversial but bears directly upon areas of human enterprise, such as ecology, agriculture, medicine, and industry (5). France is the only Western country to possess a large tropical forest heritage, with 8 million hectares of forests in its overseas territories. Most of these are tropical forests, considered to be the main world reservoir of biological diversity. In particular, French Guiana is of great interest as it has been little modified by human activities and remains largely unexplored. Tropical fungi have traditionally been underresearched, and their taxonomic placement has been confounded, often by misidentification with temperate fungi. The exploration of tropical fungi is thus limited by (i) the extensive training needed for sampling of complex tropical habitats, (ii) the paucity of newly trained systematists specializing in tropical mycology, and (iii) traditional difficulties in delineating species boundaries (3). Therefore, exploration of wood-decaying fungi is challenging.

Lignocellulose is both physically and chemically resistant to degradation even after plant death, as cellulose fibrils are embedded in the hemicellulose and lignin matrix (10). Wood-rotting fungi belonging to the phyla Basidiomycota and Ascomycota play a key role in recycling nutrients in forest ecosystems. They are known to produce a high number and broad variety of extracellular enzymes with different, complementary catalytic activities to degrade lignocellulose-rich materials (8, 9, 38). Fungal lignocellulolytic enzymes have therefore been studied for the hydrolysis of renewable biomass resources available in large amounts, such as plants, plant parts (e.g., seeds and stalks), plant constituents (e.g., starch and fiber), processing by-products (e.g., distiller's grains and corn solubles), and municipal, agricultural (e.g., straw), and industrial wastes, into high-added-value products. Bioconversion of plant cell wall polysaccharides, cellulose, and hemicellulose to simple sugars for subsequent fermentation to bioethanol has been widely studied, as the prospect of its biological production from abundant lignocellulosic feedstocks is attractive (23, 44). However, enzymatic hydrolysis (saccharification) is still the major bottleneck in the biorefinery process. The fungus *Trichoderma reesei*, an anamorph of the pantropical ascomycete *Hypocrea jecorina*, originally isolated from cotton canvas in the Solomon Islands during World War II (28), has been identified as a good candidate for monosaccharide release, due to its capacity to secrete high levels of cellulases. *T. reesei* has undergone several rounds of mutation/selection starting from the QM6a strain to increase its capacity to produce and secrete cellulases in high yields (19). As a result, the engineered *T. reesei* industrial CL847 strain is able to secrete more than 100 g of proteins per liter of culture and has been proposed to be one of the most promising strains for conversion of lignocellulose to fermentable sugars. However, conversion is still not optimal due to the heterogeneous composition of plant biomass. In addition, analysis of the *T. reesei* genome has revealed a low number and low level of diversity of enzymes likely to be involved in biomass degradation compared with the number and diversity in other filamentous fungi (24). Thus, there is a need to develop at a

Received 23 May 2012 **Accepted** 26 June 2012
Published ahead of print 6 July 2012
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doi:10.1128/AEM.01651-12

significantly reduced cost more effective enzymatic cocktails with a range of properties complementary to current cellulase systems.

In this study, fungal biodiversity in wood-decaying species was explored without *a priori* knowledge for biomass deconstruction. The strains used were isolated from fresh decaying wood specimens collected *in situ* in different geographical areas, i.e., tropical forests from French overseas territories and temperate forests from metropolitan France. Isolates were carefully identified by both morphological and molecular methods. Following production of fungal secretomes under similar inductive conditions, conversion of biomass was assessed by taking advantage of an in-house automated methodology. The potential of fungal secretomes to improve a reference cellulolytic cocktail originating from *T. reesei* was evaluated.

MATERIALS AND METHODS

Collection and identification of fungal specimens. Fruit bodies growing on dead wood were collected in 2007 and 2008 during the rainy season in both tropical areas (between April and August) and temperate areas (between October and March) in different biotopes (Table 1). Field identification of basidioma was achieved via classical methodology by macro- and micromorphological analyses using taxonomic guides and standard procedures (13, 26, 32–36). For ascoma identification, specimens were examined using the methods described by Rossman et al. (30) and Hirooka et al. (16). Microscopic observations and measurements were made in water, and ascospore ornamentation was observed in lactic cotton blue.

Isolation of strains. For each basidiomycetes specimen collected, primary isolation was made from context tissue of fresh fruit bodies on the day of collection, using malt agar medium supplemented with chloramphenicol (50 $\mu g \cdot ml^{-1}$). Apparently monomorphic cultures obtained after at least two transfers onto fresh agar plates were further authenticated using molecular tools to check strain purity and identity. With regard to ascomycetes, isolates were obtained from ascospores using potato dextrose agar (PDA; BD Difco, France) supplemented with streptomycin (5 $\mu g \cdot ml^{-1}$).

Molecular authentication (DNA extraction, PCR, and sequencing). Genomic DNA was isolated from mycelial powder (40 to 80 mg) as described by Lomascolo et al. (21). The ITS region was amplified using the ITS1 and ITS4 primers as described by White et al. (43). The ITS1-5.8S rRNA gene-ITS2 were amplified from 50 ng genomic DNA in 50 μl PCR reagent containing 1.5 U Expand High Fidelity PCR system (Roche, France) with a protocol adapted from that of Lomascolo et al. (21). Extension was carried out for 1 min at 51°C. The PCR products were sequenced by Cogenics (Meylan, France). Fungal internal transcribed spacer (ITS) sequences were checked and edited with MEGA (version 5) software (41). Using the BLAST algorithm, ITS sequences were compared with sequences in the GenBank database and FunGene-DB database (for Polyporales fungi; http://www.fungene-db.org). The best BLAST match was reported for each strain, and phylogenetic analysis was used to confirm its relevance. The taxon name was validated only when the morphological identification of the specimen and the molecular identification of the strain were consistent. All authenticated strains were deposited into the fungal culture collection of the International Centre of Microbial Resources (CIRM-CF; http://www.inra.fr/crb-cirm/) at the French National Institute for Agricultural Research (INRA; Marseille, France). The strains were maintained on malt agar (BD Difco) slants, using MA2 (malt extract at 2% [wt/vol]) medium for basidiomycetes and MYA2 (malt extract at 2% [wt/vol] and yeast extract at 0.1% [wt/vol]) medium for ascomycetes.

Culture conditions and supernatant preparation. On the basis of previous studies (6, 9, 20), the fungal cultures were grown in a liquid medium containing 15 $g \cdot liter^{-1}$ (based on the dry matter) of the autoclaved maize bran fraction (provided by ARD, Pomacle, France) as a carbon source, 2.5 $g \cdot liter^{-1}$ of maltose as a starter, 1.842 $g \cdot liter^{-1}$ of diammonium tartrate as a nitrogen source, 0.5 $g \cdot liter^{-1}$ yeast extract, 0.2 $g \cdot liter^{-1}$ KH_2PO_4, 0.0132 $g \cdot liter^{-1}$ $CaCl_2 \cdot 2H_2O$, and 0.5 $g \cdot liter^{-1}$ $MgSO_4 \cdot 7H_2O$. The sugar content (wt/wt) of the autoclaved maize bran fraction was 16.10% arabinose, 28.73% xylose, 0.17% mannose, 5.65% galactose, and 22.06% glucose. Miniaturized fungal cultures were carried out in 16-well baffled plates as described by Alberto et al. (1) and Couturier et al. (9). The cultures were inoculated with 2×10^5 spores $\cdot ml^{-1}$ for sporulating fungi or with mycelial fragments generated using a FastPrep-24 system (MP Biomedicals, France) set to 5 $m \cdot s^{-1}$ for 40 s for nonsporulating fungi, before incubation in 16-well baffled plates at 30°C with orbital shaking at 140 rpm (Infors HT, Switzerland). Each isolate was grown in 16-well baffled plates in triplicate. All the cultures were stopped at 7 and 10 days after inoculation for ascomycetes and basidiomycetes, respectively, as this was the midterm growth for the corresponding fungi under our conditions (16-well baffled plates) and extended growth led to evaporation of the culture medium. The culture broths (secretomes) were harvested and pooled (total volume, 20 to 30 ml), filtered (using a 0.2-μm-pore-size polyethersulfone membrane; Vivaspin; Sartorius, Germany), diafiltered, and concentrated (Vivaspin polyethersulfone membrane with a 10-kDa cutoff; Sartorius) in 50 mM acetate solution buffer, pH 5, to a final volume of 3 ml and then stored in appropriate vials (1.2-ml tubes with septa in the cluster plate; ABgene, Thermo Scientific) at −20°C until use.

Saccharification assays. The *T. reesei* CL847 secretome (E508 enzymatic cocktail) obtained from IFPEN (Rueil-Malmaison, France) was used as a reference enzymatic cocktail (8, 9, 40). Enzymatic commercial cocktails were obtained from the manufacturers, as indicated in Table 2. The concentrated secretomes were tested for their ability to hydrolyze micronized wheat straw (WS; *Triticum aestivum* Apache, France) that was kindly provided by F. Meaux (St. Jean du Salés, Aveyron, France), harvested in 2007, and used for grinding experiments as described by Navarro et al. (27) and Silva et al. (39). WS was stored at 4°C before use. WS particles had an average size of 100 μm. Wheat straw was composed of cellulose at 32.0 ± 0.7, hemicelluloses (arabinoxylans) at 20.5 ± 0.4, lignin at 17.4 ± 0.3, extractives at 9.5 ± 2.2, and ash at 6.1 ± 0.1 g/100 g dry wheat straw (39). A 1% (wt/vol) WS suspension was prepared in 50 mM acetate buffer, pH 5, supplemented with 40 $\mu g \cdot ml^{-1}$ of tetracycline as an antibiotic and 30 $\mu g \cdot ml^{-1}$ of cycloheximide as an antifungal agent. The resulting suspension was dispensed into 96-well plates by a Tecan Genesis Evo 200 robot (Tecan, Lyon, France), and the plates were frozen at −20°C until needed. The saccharification assay was performed by a previously described high-throughput automated method (27). All the appropriate blanks and controls were run as described by Navarro et al. (27). A substrate-free negative control was set up by filling wells with 50 mM sodium acetate buffer, pH 5, and the background of soluble sugars present in the WS sample was determined by incubating WS in the absence of enzyme. The sugars released were quantified at the saccharification plateau defined by Navarro et al. (27) as 30 μg of CL847 representing 100% sugar-releasing activity at 24 h for WS (reference activity). It is important to note that at the saccharification plateau, a supplementation of *T. reesei* CL847 enzyme cocktail by itself did not change the yield of total sugars released. To quantify the sugars released at the saccharification plateau, 15 μl of each concentrated secretome was added to the substrate plate either alone or together with 30 μg of the *T. reesei* CL847 enzymatic cocktail. The reducing sugars released were quantified using the dinitrosalicylic acid (DNS) assay (27). The addition of fungal secretomes was not normalized on the basis of the protein loading since there was a large gap in the amount of total protein secreted by fungal strains. Moreover, based on previous work (9), we noticed that the efficiency of fungal secretomes in supplementation trials is more closely correlated to enzyme diversity rather than total enzyme loading of fungal secretomes.

Enzyme activity measurements. *para*-Nitrophenyl (*p*NP)-based chromogenic substrates and complex substrates were used to assay the enzymatic activities of the *Trametes gibbosa* BRFM 952 (Banque de Ressources Fongiques de Marseille) secretome as described by Couturier et al. (9).

TABLE 1 Fungal strains collected in temperate and tropical forests and identified in this study

Forest type and taxon[d]	Classification	BRFM no.	Voucher specimen	Geographic location (country, county, city[a])	ITS GenBank accession no.
Temperate forests					
Cyanonectria buxi	Ascomycota, Nectriaceae	1205	CLL 7165	France, Deux-Sèvres	JX082385
Geejayessia sp.	Ascomycota, Nectriaceae	1015	CLL 7150	Spain, NA[b]	JX082350
Hypocrea lixii	Ascomycota, Hypocreaceae	1058	CLL 7132	France, NA	JX082359
Neonectria discophora	Ascomycota, Nectriaceae	1206	CLL 7178	France, Deux-Sèvres	JX082386
Bjerkandera adusta	Basidiomycota, Meruliaceae	965	BEL 160	France, Orne, Bellême	JX082339
Daedaleopsis confragosa	Basidiomycota, Polyporaceae	1130	BEL 08-270	France, Orne, Bellême	JX082372
Daedaleopsis confragosa	Basidiomycota, Polyporaceae	1131	BEC 08-275	France, Aude, Belcaire	JX082373
Daedaleopsis confragosa	Basidiomycota, Polyporaceae	1143	BEC 08-311	France, Aude, Bellême	JX082375
Daedaleopsis confragosa	Basidiomycota, Polyporaceae	1145	BEL 08-240	France, Orne, Bellême	JX082376
Fomitopsis pinicola	Basidiomycota, Fomitopsidaceae	882	LUM 25	France, Bouches-du-Rhône, Marseille	JX082329
Ganoderma lucidum	Basidiomycota, Ganodermataceae	885	BAU 34	France, Var, Plan d'Aups	JX082330
Ganoderma resinaceum	Basidiomycota, Ganodermataceae	872	MRS 26	France, Bouches-du-Rhône, Marseille	JX082326
Ganoderma resinaceum	Basidiomycota, Ganodermataceae	875	MRS 27	France, Bouches-du-Rhône, Marseille	JX082328
Gloeophyllum sepiarum	Basidiomycota, Gloeophyllaceae	988	ARI 08-09	France, Ariège, Varilhes	JX082348
Inonotus tamaricis	Basidiomycota, Hymenochaetaceae	880	LEC 30	France, Var, Saint-Cyr sur Mer	Only ITS1 was available
Inonotus radiatus	Basidiomycota, Hymenochaetaceae	1153	BEC 08-277	France, Aude, Roquefeuil	JX082384
Ischnoderma benzoinum	Basidiomycota, Fomitopsidaceae	1134	BEL 08-250	France, Orne, Bellême	JX082374
Merulius tremellosus	Basidiomycota, Meruliaceae	968	BEL 163	France, Orne, Bellême	JX082340
Polyporus brumalis	Basidiomycota, Polyporaceae	985	TAR 08-01	France, Hautes Pyrénées, Puydarieux	JX082346
Postia stiptica	Basidiomycota, Fomitopsidaceae	1148	HYE 08-381	France, Var, Hyères	JX082379
Postia stiptica	Basidiomycota, Fomitopsidaceae	1149	HYE 08-382	France, Var, Hyères	JX082380
Postia stiptica	Basidiomycota, Fomitopsidaceae	1150	HYE 08-386	France, Var, Hyères	JX082381
Postia stiptica	Basidiomycota, Fomitopsidaceae	1151	HYE 08-388	France, Var, Hyères	JX082382
Postia stiptica	Basidiomycota, Fomitopsidaceae	1152	POR 08-370	France, Var, Porquerolles	JX082383
Skeletocutis nivea	Basidiomycota, Polyporaceae	987	ND 167	France, Pyrénées Atlantiques, Correze	JX082347
Stereum hirsutum	Basidiomycota, Stereaceae	889	BAU 42	France, Var, Plan d'Aups	JX082331
Lenzites warnieri	Basidiomycota, Polyporaceae	972	ND 169	France, Vaucluse, Lumières	JX082341
Lenzites warnieri	Basidiomycota, Polyporaceae	973	ND 181	France, Vaucluse, Opedette	JX082342
Trametes gibbosa	Basidiomycota, Polyporaceae	873	BEL 2	France, Orne, Bellême, Beech tree	JX082327
Trametes gibbosa	Basidiomycota, Polyporaceae	952	MOU 147	France, Ariège, Moulis, Beech Forest	JX082338
Trametes gibbosa	Basidiomycota, Polyporaceae	983	ARI 08-03	France, Ariège, Varilhes	JX082344
Trametes gibbosa	Basidiomycota, Polyporaceae	1115	BEL 08-268	France, Orne, Bellême	JN645064[c]
Trametes gibbosa	Basidiomycota, Polyporaceae	1147	BEL 08-255	France, Orne, Bellême	JX082378
Trametes hirsuta	Basidiomycota, Polyporaceae	984	LYO 08-10	France, Rhône, Rontalon	JX082345
Trametes ochracea	Basidiomycota, Polyporaceae	1019	DRO 08-12	France, Drôme, Bourg de Péage	JX082351
Trametes sp. (*Trametes* aff. *meyenii*)	Basidiomycota, Polyporaceae	1121	GUY 08-152	French Guiana, Macouria	JN645083[c]
Trametes versicolor	Basidiomycota, Polyporaceae	1116	BEL 08-252	France, Orne, Bellême	JX082367
Trametes versicolor	Basidiomycota, Polyporaceae	1146	BEL 08-253	France, Orne, Bellême	JX082377
Tropical forests					
Nectria pseudocinnabarina	Ascomycota, Nectriaceae	1288	CLL 8299	Martinique, NA	JX082392
Hypocrea lixii	Ascomycota, Hypocreaceae	1285	CLL 110	French Guiana, Nouragues	JX082390
Neocosmospora cf. *hematococca*	Ascomycota, Nectriaceae	1214	CLL 8033	French Guiana, Sinnamary	JX082388
Neocosmospora cf. *hematococca*	Ascomycota, Nectriaceae	1286	CLL 8012	French Guiana, Kourou	JX082391
Rugonectria cf. *rugulosa*	Ascomycota, Nectriaceae	1213	CLL 8212	Guadeloupe, Capesterre-Belle-Eau	JX082387
Xylaria curta	Ascomycota, Xylariaceae	1269	CLL 8045	French Guiana, Sinnamary	JX082389
Amauroderma sp.	Basidiomycota, Ganodermataceae	915	GUY 74	French Guiana, Sinnamary	JX082335
Amauroderma sp.	Basidiomycota, Ganodermataceae	916	GUY 83	French Guiana, Sinnamary	JX082336
Amauroderma sp.	Basidiomycota, Ganodermataceae	1117	GUY 08-151	French Guiana, Macouria	JX082393
Artolenzites elegans (*Trametes elegans*)	Basidiomycota, Polyporaceae	1122	GUY 08-145	French Guiana, Saül	JN645066[c]
Coriolopsis sp.	Basidiomycota, Polyporaceae	1125	GUY 08-85	French Guiana, Sinnamary	JX082370
Coriolopsis sp.	Basidiomycota, Polyporaceae	1126	GUY 08-201	French Guiana, Macouria	JX082371
Cyathus sp.	Basidiomycota, Agaricaceae	934	GUY 104	French Guiana, Sinnamary	JX082337
Earliella scabrosa	Basidiomycota, Polyporaceae	1106	GUY 08-137	French Guiana, Saül	JX082364
Fomes fasciatus	Basidiomycota, Polyporaceae	1081	GUY 08-34	French Guiana, Kourou	JX082362
Ganoderma sp.	Basidiomycota, Ganodermataceae	1030	GUY 08-48	French Guiana, Sinnamary	JX082353
Ganoderma sp.	Basidiomycota, Ganodermataceae	1035	GUY 08-107	French Guiana, Saül	JX082354
Ganoderma subfornicatum	Basidiomycota, Ganodermataceae	1024	GUY 08-57	French Guiana, Sinnamary	JX082352

(Continued on following page)

TABLE 1 (Continued)

Forest type and taxon[d]	Classification	BRFM no.	Voucher specimen	Geographic location (country, county, city[a])	ITS GenBank accession no.
Grammothele sp.	Basidiomycota, Polyporaceae	910	GUY 82	French Guiana, Sinnamary	JX082334
Gymnopilus sp.	Basidiomycota, Strophariaceae	1082	GUY 08-73	French Guiana, Kourou	JX082363
Lenzites sp.	Basidiomycota, Polyporaceae	1048	GUY 08-17	French Guiana, Kourou	JX082355
Lenzites sp.	Basidiomycota, Polyporaceae	1049	GUY 08-18	French Guiana, Kourou	JX082356
Lenzites sp. (*Leiotrametes* sp.)	Basidiomycota, Polyporaceae	1050	GUY 08-20	French Guiana, Kourou	GU731566[c]
Lenzites sp.	Basidiomycota, Polyporaceae	1053	GUY 08-134	French Guiana, Saül	JX082357
Lenzites sp.	Basidiomycota, Polyporaceae	1054	GUY 08-146	French Guiana, Saül	JX082358
Lenzites sp. (*Leiotrametes* sp.)	Basidiomycota, Polyporaceae	1078	GUY 08-156	French Guiana, Macouria	JN645062[c]
Lenzites sp.	Basidiomycota, Polyporaceae	1079	GUY 08-159	French Guiana, Macouria	JX082361
Lenzites sp. (*Leiotrametes* sp.)	Basidiomycota, Polyporaceae	1080	GUY 08-167	French Guiana, Kourou	JN 645063[c]
Phellinus sp.	Basidiomycota, Hymenochaetaceae	907	GUY 33	French Guiana, Sinnamary	JX082332
Phellinus sp.	Basidiomycota, Hymenochaetaceae	908	GUY 70	French Guiana Sinnamary	JX082333
Phellinus sp.	Basidiomycota, Hymenochaetaceae	1007	GUY 07-05	French Guiana, Sinnamary	JX082349
Pycnoporus sanguineus	Basidiomycota, Polyporaceae	981	CAL 189	New Caledonia, NA	JX082343
Pycnoporus sanguineus	Basidiomycota, Polyporaceae	1114	GUY 08-215	French Guiana, Cayenne	JX082366
Tinctoporellus epimiltinus	Basidiomycota, Polyporaceae	1077	GUY 08-26	French Guiana, Kourou	JX082360
Trametes lactinea (*Leiotrametes lactinea*)	Basidiomycota, Polyporaceae	1119	GUY 08-16	French Guiana, Kourou	JX082368
Trametes lactinea (*Leiotrametes lactinea*)	Basidiomycota, Polyporaceae	1120	GUY 08-131	French Guiana, Saül	JX082369

[a] When available.
[b] NA, not available.
[c] Welti et al. (42).
[d] cf. and aff. indicate members of the indicated genus resembling the listed species.

Nucleotide sequence accession numbers. All the nucleotide sequences were deposited in GenBank under the accession numbers given in Table 1.

RESULTS

Fungal diversity. Whatever the climatic zone (tropical or temperate), about half of the isolates did not grow on plates and/or were contaminated by other microorganisms (data not shown). A total of 74 pure cultures was finally recovered (Table 1), from which 69% of the isolates were identified to the species level (34 different species mainly from temperate regions) and 31% were identified to the genus level only (mainly from tropical regions). A cladogram, constructed using ITS sequences, illustrates the fungal diversity thus obtained (Fig. 1). Among the fungal species recovered and authenticated, 64 belonged to basidiomycetes mainly from the *Polyporaceae*, *Ganodermataceae*, *Fomitopsidaceae*, and *Hymenochaetaceae* and 10 belonged to ascomycetes mainly from the *Nectriaceae*.

TABLE 2 Fungal commercial cocktails tested[a]

Enzyme cocktail	Origin	Fungal species	% sugar	
			−CL847	+CL847
E508-CL847	IFPEN	*Trichoderma reesei*	100	100
Accelerase 1500	Genencor	*Trichoderma reesei*	91	113
Cellobiase 188	Novozyme	*Aspergillus niger*	31	113
Celluclast	Novozyme	*Trichoderma reesei*	98	117
Depol 686L	Biocatalysts	*Trichoderma reesei*	98	104
Depol 740L	Biocatalysts	*Humicola* sp.	41	119
Hemicellulase	Sigma	*Aspergillus niger*	38	115
Pectinex Ultra SP-L	Novozyme	*Aspergillus aculeatus*	62	102
Viscozyme L	Novozyme	*Aspergillus aculeatus*	4	102
Xylanase	Sigma	*Trichoderma viride*	39	113

[a] Total solubilized sugars were measured using enzyme cocktails alone (−CL847) and supplemented with the *T. reesei* secretome (+CL847) and were expressed as a percentage by taking the result for *T. reesei* CL847 commercial cocktail as a reference. Values are means of at least triplicate measures performed independently. Standard errors of the means were <5%.

Production of fungal secretomes. Hydrolysis of plant biomass requires the concerted action of a range of lignocellulolytic enzymes. The secretion of these enzymes *in vitro* is regulated by the type and complexity of the plant material used as an inducer for the fungal cultures. In this study, maize bran was selected as it is a powerful inducer for the expression of a broad range of genes that encode lignocellulolytic enzymes, e.g., endoxylanases, endomannanases, arabinofuranosidases, carbohydrate esterases, and oxidoreductases, as previously shown by Couturier et al. (9). All the fungi tested were able to grow on maize bran with a satisfactory yield of secreted protein and were harvested at a single time point, i.e., 7 days of growth for ascomycetes and 10 days of growth for basidiomycetes (see Materials and Methods). All secretomes were similarly diafiltered and then tested individually for their ability to release sugars from biomass, taking advantage of our in-house automated saccharification method (27). Given the crops produced in France for possible conversion to biofuel, we focused our study on cereal straw from wheat.

Saccharification efficiencies of fungal secretomes. The release of reducing sugars from wheat straw (WS) was quantified at the saccharification plateau (24 h), taking the *T. reesei* CL847 enzymatic cocktail as a reference (Table 3). Experiments were performed using fungal secretomes either alone or in combination with the *T. reesei* CL847 enzymatic cocktail. Data were fitted using a quartile statistical analysis.

The best-performing secretomes alone were represented by quartile 4, which comprised 6 ascomycetes, including four species of *Nectriaceae* and two species of *Hypocrea lixii* (teleomorph of *Trichoderma harzianum*) and 13 basidiomycetes with 6 fungi of

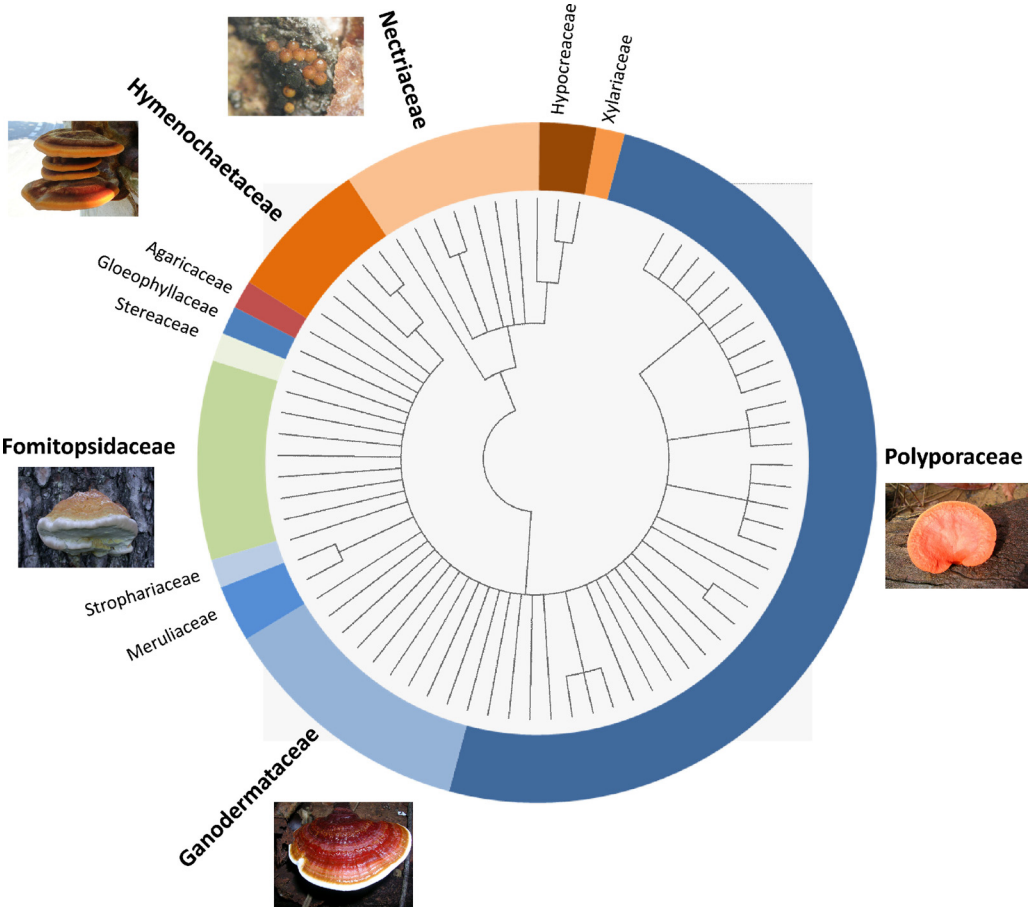

FIG 1 Natural fungal diversity explored. The cladogram was constructed on the basis of ITS sequences, and pictures illustrate the main fungal classes represented.

temperate origin and 13 of tropical origin. We noted a significant difference in effectiveness among strains of the genus *Ganoderma* according to geographical origin. Among the six *Ganoderma* spp., the three of tropical origin were present in quartile 4 (best performing) and the three of temperate origin were in quartile 1 (worst performing). For comparison, the commercial cocktails originating from fungi were tested under the same experimental conditions (Table 2). The results comparing fungal secretome and commercial cocktails alone are shown in Fig. 2. The most efficient commercial cocktails all originated from *Trichoderma* strains (CL847-E508, Depol 686L, Celluclast, and Accellerase 1500) with yields of between 91% and 100%. It is striking that three fungi (*Ganoderma* sp. strain BRFM 1030, *Artolenzites elegans* BRFM 1122, and *Hypocrea lixii* BRFM 1285) in quartile 4 yielded amounts of sugars comparable to those of *T. reesei* commercial cocktails, with, respectively, 87%, 84% and 84%.

Each fungal secretome was also tested in combination with *T. reesei* CL847 enzyme cocktail (Table 3). Of the 19 fungi that performed best under our experimental conditions (above 123%, corresponding to quartile 4), only 4 were ascomycetes (two species of *Nectriaceae* and two *Hypocrea lixii* isolates), whereas all the other strains were basidiomycetes (e.g., the genera *Daedaleopsis*, *Phellinus*, and *Trametes*). The three *Daedaleopsis confragosa* isolates (BRFM 1131, BRFM 1143, and BRFM 1145) were classified in quartile 4. Two fungal secretomes belonging to the *Trametes*-related genera (*Lenzites* sp. strain BRFM 1048 from tropical forests and *Trametes gibbosa* BRFM 952 from temperate forests) stood out from the others by improving the conversion yield by 53% and 60%, respectively (Table 3 and Fig. 2). In quartile 4, no correlation in relation with geographical distribution was observed, i.e., 8 isolates collected in temperate forests and 10 collected in tropical forests. For comparison, the fungal commercial cocktails tested under the same experimental conditions failed to efficiently supplement the *T. reesei* CL847 cocktail (Table 2; Fig. 2).

Enzymatic characterization of the *Trametes gibbosa* BRFM 952 secretome. As the secretome of *T. gibbosa* BRFM 952 was the best performing one in combination with the *T. reesei* CL847 commercial cocktail (Table 3; Fig. 2), we assessed its sugar-cleaving capabilities. We quantified its main glycoside hydrolase activities using a microplate assay that contained *p*NP sugars and complex polysaccharides as the substrates (Table 4). Cellulose degradation was estimated by the quantification of endoglucanase (carboxymethyl cellulose [CMC]), cellulase (Avicelase; Avicel [AVI]), filter paper (FP), cellobiohydrolase (*p*NP-β-D-cellobioside [pCel] and *p*NP-β-D-lactobioside [pLac]), and β-glucosidase (*p*NP-β-D-glucopyranoside [pGlu]) activities. Although the *T. gibbosa* BRFM 952 secretome did not display any cellobiohydrolase activity, it showed activity 8 times higher on crystalline cellulose (AVI) than the *T. reesei* CL847 enzymatic cocktail. Also, the overall activity on cellulose of the *T. gibbosa* BRFM 952 secretome measured using FP

TABLE 3 Contribution of fungal secretomes to the saccharification of wheat straw[a]

Species	BRFM no.	−CL847		+CL847	
		% sugar	Quartile	% sugar	Quartile
Ganoderma sp.	1030	87	**4**	108	1
Artolenzites elegans	1122	84	**4**	121	3
Hypocrea lixii	1285	84	**4**	127	**4**
Trametes gibbosa	952	74	**4**	160	**4**
Hypocrea lixii	1058	65	**4**	137	**4**
Coriolopsis sp.	1126	61	**4**	95	1
Neonectria discophora	1206	60	**4**	114	2
Nectria pseudocinnabarina	1288	56	**4**	134	**4**
Rugonectria cf. *rugulosa*	1213	55	**4**	112	2
Cyanonectria buxi	1205	53	**4**	131	**4**
Tinctoporellus epimiltinus	1077	52	**4**	132	**4**
Ganoderma subfornicatum	1024	51	**4**	113	2
Trametes ochracea	1019	50	**4**	124	**4**
Lenzites sp.	1054	46	**4**	123	**4**
Postia stiptica	1152	46	**4**	117	3
Phellinus sp.	1007	45	**4**	125	**4**
Trametes sp.	1121	45	**4**	105	1
Coriolopsis sp.	1125	44	**4**	113	2
Ganoderma sp.	1035	44	**4**	117	3
Daedaleopsis confragosa	1143	43	3	127	**4**
Daedaleopsis confragosa	1145	43	3	135	**4**
Trametes gibbosa	1147	42	3	114	2
Merulius tremellosus	968	41	3	120	3
Postia stiptica	1148	41	3	119	3
Skeletocutis nivea	987	41	3	91	1
Trametes lactinea	1119	41	3	95	1
Trametes lactinea	1120	41	3	114	2
Postia stiptica	1150	40	3	114	2
Postia stiptica	1151	40	3	116	3
Daedaleopsis confragosa	1131	39	3	123	**4**
Lenzites sp.	1053	39	3	112	2
Trametes gibbosa	1115	39	3	104	1
Trametes versicolor	1146	39	3	128	**4**
Amauroderma sp.	1117	38	3	112	2
Amauroderma sp.	916	38	3	127	**4**
Earliella scabrosa	1106	38	3	120	3
Phellinus sp.	907	38	3	118	3
Daedaleopsis confragosa	1130	37	2	111	2
Geejayessia sp.	1015	37	2	121	3
Inonotus radiatus	1153	37	2	114	2
Ischnoderma benzoinum	1134	37	2	121	3
Lenzites sp.	1079	36	2	112	2
Postia stiptica	1149	36	2	108	1
Trametes gibbosa	983	36	2	96	1
Xylaria curta	1269	36	2	101	1
Pycnoporus sanguineus	1114	35	2	128	**4**
Trametes versicolor	1116	35	2	103	1
Ganoderma lucidum	885	34	2	115	3
Gymnopilus sp.	1082	34	2	111	2
Lenzites sp.	1048	34	2	153	**4**
Lenzites warnieri	972	33	2	113	2
Neocosmospora cf. *hematococca*	1214	33	2	126	**4**
Phellinus sp.	908	33	2	123	**4**
Fomes fasciatus	1081	32	2	108	1
Neocosmospora cf. *hematococca*	1286	32	2	121	3
Lenzites sp.	1049	30	1	111	2
Fomitopsis pinicola	882	29	1	98	1
Inonotus tamaricis	880	29	1	122	3
Lenzites sp.	1050	29	1	110	2
Lenzites sp.	1078	29	1	117	3
Lenzites warnieri	973	28	1	103	1
Trametes hirsuta	984	28	1	122	**4**
Bjerkandera adusta	965	27	1	118	3
Pycnoporus sanguineus	981	27	1	95	1
Gloeophyllum sepiarum	988	26	1	117	3
Polyporus brumalis	985	26	1	106	1
Amauroderma sp.	915	25	1	112	2
Lenzites sp.	1080	25	1	120	3
Trametes gibbosa	873	24	1	84	1
Cyathus sp.	934	22	1	104	1
Grammothele sp.	910	22	1	103	1
Ganoderma resinaceum	875	20	1	110	2
Ganoderma resinaceum	872	17	1	102	1
Stereum hirsutum	889	0	1	117	3

[a] Total solubilized sugars were measured using fungal secretomes alone (−CL847) and in combination with the *T. reesei* secretome (+CL847) and are expressed in percent by taking the *T. reesei* CL847 commercial cocktail as a reference. Values are means of at least triplicate measures performed independently. Standard errors of the means were <5%. Results are classified into four categories using a quartile statistical analysis: for fungal secretomes tested alone, quartile 1 ≤ 31, 32 ≤ quartile 2 ≤ 37, 38 ≤ quartile 3 ≤ 43, and quartile 4 ≥ 44; for fungal secretomes tested in supplementation, quartile 1 ≤ 109, 110 ≤ quartile 2 ≤ 114, 115 ≤ quartile 3 ≤ 122, and quartile 4 ≥ 123. Quartile 4 is highlighted in bold.

was twice that of *T. reesei* CL847. Hemicellulose degradation was estimated by quantifying the xylanase activities using structurally different xylans (birchwood xylan [BRX], soluble wheat arabinoxylan [SAX], and insoluble wheat arabinoxylan [IAX]) as the substrates, and the exo-acting glycosidase activities were estimated using *p*NP-α-L-arabinofuranoside (pAra) and *p*NP-β-D-xylopyranoside (pXyl). The *T. gibbosa* BRFM 952 secretome displayed some hemicellulase activities, but they were not higher than the hemicellulase activity of the *T. reesei* CL847 cocktail. Pectic degradation was assessed using pectin, arabinogalactan (AGA), arabinan (ARB), and *p*NP-α-D-galactopyranoside (pGal). We note that pectin-related activities were present in significantly smaller amounts in the commercial cocktail than in the *T. gibbosa* BRFM 952 secretome. The overall esterase activity assessed using *p*NP-acetate (pAc) was not detected in the *T. gibbosa* BRFM 952 or *T. reesei* CL847 secretome. We also note that 4.39 U of laccase activity per mg of protein was measured in the *T. gibbosa* BRFM 952 secretome, whereas no activity was detected in the *T. reesei* CL847 cocktail.

DISCUSSION

Our study is the first to report large-scale trials of supplementation of a commercial enzymatic cocktail with secretomes from fungal strains isolated from natural diversity. In addition, the current literature contains only sparse data on the screening of fungal strains using real lignocellulosic materials. Shrestha et al. (37) have isolated novel fungal species (mainly ascomycetes) from decaying bioenergy grasses, among which some were able to convert *Miscanthus* biomass. However, the dry weight loss was only 8 to 13% over 4 weeks. Recently, there has also been growing interest in the potential of plant-pathogenic fungi to optimize hydrolysis of lignocellulosic biomass (12). For instance, large-scale screening of ascomycetes revealed that the plant pathogens were more active than the nonpathogens on several lignocellulosic substrates (17).

From the 74 fungi identified and tested in this study, we found both ascomycetes and basidiomycetes strains that significantly improve sugar conversion in combination with the reference *T. reesei* CL847 commercial cocktail. None of the other fungal commercial cocktails tested under the same experimental conditions resulted in any significant increase in biomass conversion, although they contained a large range of carbohydrate-active enzyme (CAZyme) activities (7; www.cazy.org). The fungi that best performed alone (*Ganoderma* sp. strain BRFM 1030, *Artolenzites elegans* BRFM 1122, and *Hypocrea lixii* BRFM 1285) were not the

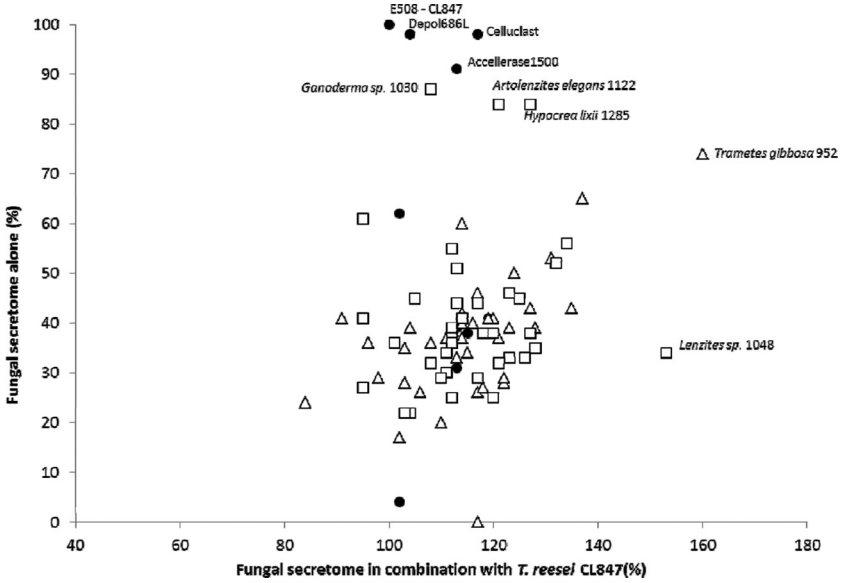

FIG 2 Species distribution following hydrolysis of biomass. Hydrolysis of biomass was performed with fungal secretomes alone (*y* axis) and in supplementation with the *T. reesei* commercial cocktail (*x* axis). Fungal secretomes were from commercial cocktails (full circles), tropical isolates (open squares), or temperate isolates (open triangles). Names of the best-performing fungi and commercial cocktails are indicated.

best-performing ones when tested in combination with the *T. reesei* CL847 enzymatic cocktail. This suggests that their enzymatic strategies to deconstruct lignocellulose were similar to those *T. reesei*. Among the best-performing fungal secretomes that significantly supplemented the *T. reesei* enzymatic cocktail (i.e., quartile 4), we found several genera belonging to the white-rot fungi (e.g., the genera *Daedaleopsis*, *Phellinus*, *Pycnoporus*, and *Trametes*). White-rot fungi mineralize cell wall components (cellulose, hemicelluloses, and lignins) and extensively degrade lignins (38). For instance, *Pycnoporus* is known to produce high FP endoglucanase, β-glucosidase, xylanase, mannanase, α-galactosidase, α-arabinofuranosidase, and polygalacturonase activities (11) as well as large amounts of laccases (22) and cellobiose dehydrogenase (CDH) (4), which have been shown to affect biomass conversion (4, 11, 25). It is now clear that fungi convert lignocellulose through a multienzymatic process involving numerous CAZymes and oxidative enzymes.

The *Trametes gibbosa* BRFM 952 was the best-performing isolate with a 60% improved conversion, a feature that was not universal to the *Trametes* and related genera. Indeed, some *Trametes* isolates (e.g., *T. gibbosa* BRFM 873 and *T. versicolor* BRFM 1116) were classified in quartile 1 (i.e., corresponding to the worst-performing secretomes). Variability in biomass conversion suggests that enzymatic pools are different following induction of the secretion of lignocellulose-acting enzymes due to genetic differences among the organisms. This feature was observed by King et al. (17) and Russell et al. (31). They indicate that after the identification of promising species, there is still significant variation among isolates which may reveal superior candidates. It thus highlights the importance of a systematic high-throughput bioconversion assessment of collected fungal biodiversity in order to identify promising fungal isolates, particularly if a genus or species is suspected of being hypervariable. The increase in sugar conversion obtained when the *T. reesei* enzymatic cocktail was supplemented with the *T. gibbosa* BRFM 952 secretome could be explained by the presence of CAZymes or oxidoreductases absent in *T. reesei*. The *T. gibbosa* BRFM 952 secretome displayed a high activity on cellulose, in close agreement with the findings of Couturier et al. (9), with FP activity and WS conversion being correlated following analysis of a set of 20 fungi using activity profiling. Recent data from the literature on the synergy between family GH61 enzymes (copper-dependent polysaccharide monooxygenases) and oxidoreductases acting on lignocellulose components (18) could also explain the efficiency of the *T. gibbosa* secretome in combination with the *T. reesei* CL847 enzymatic cocktail, which contains an enzyme from the family GH61 (9).

Obviously, exploration of fungal biodiversity through their secretomes is currently one of the most relevant methods to find new enzymes of industrial interest involved in lignocellulose degradation (9, 29). Any of the top candidates identified in this study would be ideal for closely controlled synergy experiments in fu-

TABLE 4 Lignocellulosic enzyme activities of *T. reesei* enzyme preparation E508 (CL847 strain) and *Trametes gibbosa* BFRM 952 secretome

	Lignocellulosic enzyme activity ($U \cdot mg^{-1}$)																
	Cellulose-degrading enzymes						Xylan-degrading enzymes					Pectin-degrading enzymes				Others	
Secretome source	FP	AVI	CMC	pGlu	pLac	pCel	BRX	SAX	IAX	pXyl	pAra	Pectin	ARB	AGA	pGal	pAc	Laccase
E508 (CL847)	0.12	0.01	0.33	0.19	0.04	0.05	0.94	1.59	0.37	0.01	0.02	0.01	0.01	0.01	0.01	ND[a]	ND
T. gibbosa BRFM 952	0.24	0.08	0.04	0.03	ND	ND	0.18	0.29	0.10	0.01	0.02	0.28	0.08	0.47	ND	ND	4.39

[a] ND, no activity detected.

ture work. The use of proteomics is the next step for these explorations, together with transcriptomics for the dynamic study of enzyme production. A full exploitation of the data requires access to their genomic information, which will be a major breakthrough to gain a better understanding of this fungal biodiversity.

ACKNOWLEDGMENTS

This study was funded by the French National Research Agency (ANR; program E-TRICEL ANR-07-BIOE-006).

We thank X. Rouau and G. Ghizzi for the preparation of micronized wheat straw and Genencor and Biocatalysts for providing samples of commercial cocktails. Fungi were collected during field trips organized in metropolitan France, French Guiana, Guadeloupe, and Martinique. We thank the UMR EcoFog, the DIREN of Guadeloupe and Martinique, the ONF Martinique, and the Parc National de Guadeloupe administration for their assistance. The authors are especially grateful to H. Charlotte, mayor of Saül and president of the Parc National Amazonien de Guyane, for his help during our stay in this locality. B. Rivoire is acknowledged for morphological identification of *Postia stiptica*.

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