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BIODEGRADATION

Polyporales Brown Rot Species *Fomitopsis pinicola*: Enzyme Activity Profiles, Oxalic Acid Production, and Fe^{3+}-Reducing Metabolite Secretion

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ABSTRACT Basidiomycota fungi in the order Polyporales are specified to decomposition of dead wood and woody debris and thereby are crucial players in the degradation of organic matter and cycling of carbon in the forest ecosystems. Polyporales wood-decaying species comprise both white rot and brown rot fungi, based on their mode of wood decay. While the white rot fungi are able to attack and decompose all the lignocellulose biopolymers, the brown rot species mainly cause the destruction of wood polysaccharides, with minor modification of the lignin units. The biochemical mechanism of brown rot decay of wood is still unclear and has been proposed to include a combination of nonenzymatic oxidation reactions and carbohydrate-active enzymes. Therefore, a linking approach is needed to dissect the fungal brown rot processes. We studied the brown rot Polyporales species *Fomitopsis pinicola* by following mycelial growth and enzyme activity patterns and generating metabolites together with Fenton-promoting Fe^{3+}-reducing activity for 3 months in submerged cultures supplemented with spruce wood. Enzyme activities to degrade hemicellulose, cellulose, proteins, and chitin were produced by three Finnish isolates of *F. pinicola*. Substantial secretion of oxalic acid and a decrease in pH were notable. Aromatic compounds and metabolites were observed to accumulate in the fungal cultures, with some metabolites having Fe^{3+}-reducing activity. Thus, *F. pinicola* demonstrates a pattern of strong mycelial growth leading to the active production of carbohydrate- and protein-active enzymes, together with the promotion of Fenton biochemistry. Our findings point to fungal species-level "fine-tuning" and variations in the biochemical reactions leading to the brown rot type of wood decay.

IMPORTANCE *Fomitopsis pinicola* is a common fungal species in boreal and temperate forests in the Northern Hemisphere encountered as a wood-colonizing saprotroph and tree pathogen, causing a severe brown rot type of wood degradation. However, its lignocellulose-decomposing mechanisms have remained undiscovered. Our approach was to explore both the enzymatic activities and nonenzymatic Fenton reaction-promoting activities (Fe^{3+} reduction and metabolite production) by cultivating three isolates of *F. pinicola* in wood-supplemented cultures. Our findings on the simultaneous production of versatile enzyme activities, including those of endoglucanase, xylanase, β-glucosidase, chitinase, and acid peptidase, together with generation of low pH, accumulation of oxalic acid, and Fe^{3+}-reducing metabolites, increase the variations of fungal brown rot decay mechanisms. Furthermore, these findings will aid us in revealing the wood decay proteomic, transcriptomic, and metabolic activities of this ecologically important forest fungal species.

KEYWORDS Agaricomycetes, Fenton reaction, Polyporales, basidiomycetes, biodegradation, brown rot, fungal enzymes, lignocellulose, oxalic acid, wood decay fungi

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The order Polyporales is a diverse group of fungi belonging to the Basidiomycota class Agaricomycetes, containing many efficient wood decay species which are capable of degrading a range of lignocelluloses (1). The Polyporales are mainly saprotrophic wood decay fungi, including a few species of plant pathogens (2). Two major types of wood decay are recognized among Polyporales: white rot decay that modifies and degrades lignin units and the polymeric polysaccharides (cellulose and hemicelluloses) of wood, and brown rot decay that decomposes wood cellulose and hemicelluloses by a combination of nonenzymatic oxidation via Fenton chemistry and secreted carbohydrate-active enzymes (CAZymes) (3–9).

In this study, we describe enzymatic and biochemical activities generated by the brown rot Polyporales species *Fomitopsis pinicola* (Swartz:Fr.) Karst., a wood-inhabiting and bracket-forming fungus. The species has a worldwide distribution around temperate and boreal forests, being also abundant in the forests of Finland and northern Scandinavia, and it causes distinctive brown-colored wood decay in conifers and hardwoods (10). The sequenced and annotated genome of *F. pinicola* demonstrates the presence of a set of genes encoding CAZymes and auxiliary oxidoreductases (6), thereby suggesting their role in the lignocellulose-degrading system of the fungus. However, the production of wood decay enzyme activities in connection to the brown rot-promoting Fenton reactions has not been studied for the species before. Moreover, genetic studies have indicated variation among populations of the species (10), which suggests differences in biochemical characteristics between isolates.

In brown rot fungi, the proposed universal two-step process of wood decay involves hydroxyl radical-mediated oxidation of lignocellulose via a Fenton reaction, followed by the enzymatic degradation of cellulose and hemicellulose (9, 11–14). The generation of hydroxyl radicals via a Fenton reaction requires active reduction of Fe^{3+} ions to Fe^{2+}, acidic conditions, and supply of hydrogen peroxide (14). The brown rot oxidation process and generation of hydroxyl radicals have been proposed to be spatially and/or temporally segregated from fungus-secreted hydrolytic CAZymes in order to limit enzyme inactivation and protein destruction by hydroxyl radicals (15, 16).

Brown rot fungi secrete oxalic acid in significant quantities, chelating Fe^{3+} in the vicinity of hyphae at low pH (<3), which restrains the reduction of Fe^{3+} and initiation of Fenton chemistry at the site (15–17). A decreasing gradient of oxalic acid concentration by diffusion away from the hyphae with subsequent increase in pH will result in the dissociation of Fe^{3+}-oxalate chelates at the wood cell walls, thus initiating Fenton reactions. Metabolites (low-molecular-weight reductants) secreted by the fungus or similar compounds released from the woody matrix are considered to participate in the reactions by reducing Fe^{3+} to Fe^{2+} ions, thereby fueling Fenton reaction and oxidative attack in wood, leading to an opening up of the compact lignocellulose matrix (9, 12, 15, 17).

The Fenton-based oxidative system may enhance enzymatic degradation by exposing the composite fine structure of wood cell walls composed of cellulose microfibrils, hemicellulose, and lignin units to the lignocellulose-attacking CAZymes secreted by the fungus (9). Comparative genomic analyses and evolutionary studies of wood-decaying Agaricomycetes and Polyporales fungi have demonstrated genetic factors directing white or brown rot decay mechanisms, for instance, the lack of lignin-modifying class II peroxidases in brown rot species (1, 5–7, 18). However, hemicellulose- and cellulose-active enzymes have been detected in a few brown rot species, with suggestions that hemicelluloses may be decomposed prior to cellulose degradation upon brown rot decay (19–22). Therefore, together with nonenzymatic Fenton reactions, CAZyme xylanases, endoglucanases, and glucosidases form a major part of the brown rot decay system targeted to these components of wood lignocellulose and the resulting saccharides (4, 18, 23).

Uncovering the brown rot decay mechanisms using sophisticated biochemical analyses and biomimetics (9, 13–16, 24) or by omics studies that include extensive genome sequencing and proteomics (1, 5, 6, 18) has turned out to be more complicated than expected. Thus, more combinatory approaches are needed in order to

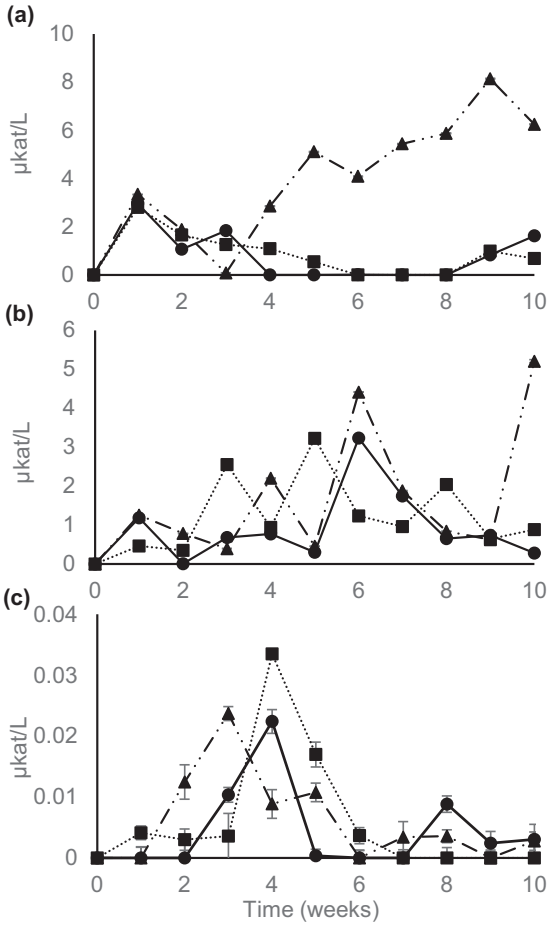

FIG 1 Extracellular enzyme activities of *Fomitopsis pinicola* against hemicellulose and cellulose. Endo-β-1,4-xylanase (a), endo-β-1,4-glucanase (b), and β-glucosidase (c) activities were detected from wood-LNAS cultures during 10 weeks of cultivation. *F. pinicola* isolates FBCC1181 (dotted line, closed square), FBCC1243 (long dash-dot, closed triangle), and FBCC1468 (continuous line, closed circle) are shown. Bars indicate the standard error (SE) ($n = 5$, biological replicates). In some cases, the error bar is not visible due to small deviation values.

determine the details of brown rot decay. Due to differences in gene composition and proteome-secretome characteristics between species of brown rot fungi (1, 2, 5, 6, 18), it may well be assumed that there are variations in the regulation and initiation of the nonenzymatic Fenton reactions versus enzymatic activities among Polyporales fungi.

In this study, we focused on the species *Fomitopsis pinicola* due to its ecological significance in forest ecosystems and the scarcity of biochemical evidence of its brown rot mechanism. Three Finnish isolates of *F. pinicola* presented notable CAZyme activities against hemicellulose and cellulose, as well as enzymatic degradation of chitin and proteins, together with release of Fe^{3+}-reducing activity and a substantial accumulation of oxalic acid leading to an increase in extracellular acidity. Moreover, fungus-generated extracellular compound fractions were observed to demonstrate Fe^{3+}-reducing activity, thus being potential candidates for more detailed studies on wood-active metabolites and oxidative Fenton reactions in lignocellulose bioconversions.

RESULTS

Carbohydrate-active enzyme production profiles. The three isolates of *F. pinicola* exhibited hemicellulose- and cellulose-degrading CAZyme activities on the low-nitrogen asparagine-succinate with spruce wood (wood-LNAS) medium, and endo-β-1,4-xylanase (xylanase), endo-β-1,4-glucanase (EG), and β-glucosidase (BG) activities were detected (Fig. 1). Extracellular laccase and peroxidase activities were not detected

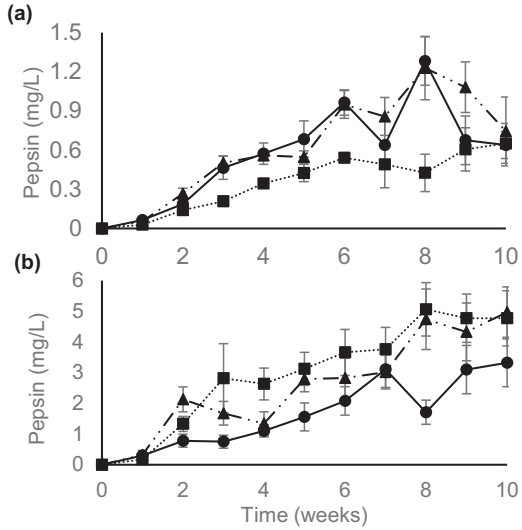

FIG 2 Extracellular acidic protease activity of *F. pinicola* isolates on wood-LNAS (a) and ME (b) media during 10 weeks of cultivation. Isolates shown are FBCC1181 (dotted line, closed square), FBCC1243 (long dash-dot, closed triangle), and FBCC1468 (continuous line, closed circle). Peptidase activity is represented as the activity generated by the amount of pepsin equivalent in milligrams per liter of culture filtrate. Bars indicate SE ($n = 5$, biological replicates). In some cases, the error bar is not visible due to small deviation values.

in the culture fluid samples (data not shown), which is in accordance with the findings of our recent study on the *F. pinicola* FBCC1181 isolate enzyme activity profile under similar medium and cultivation conditions (25).

All three *F. pinicola* isolates demonstrated xylanase activity peaking within the first week of cultivation on wood-LNAS medium, which was then followed by a decline (Fig. 1a). After 4 weeks, however, dissimilarities were observed in xylanase activity profiles between the three isolates. While the isolate *F. pinicola* FBCC1243 demonstrated the highest xylanase activity of 8.14 μkat/liter observed around cultivation week 9, the two other isolates showed very low activity levels (Fig. 1a). EG activity was also detected with all isolates on wood-LNAS medium, with a fluctuating pattern (Fig. 1b). Low levels of BG activity were observed with all isolates on wood-LNAS medium, with activities peaking either on the third or fourth week of cultivation (Fig. 1c). A potential second phase for BG activity was observed with two isolates during cultivation weeks 7 to 9.

Proteolytic and chitinolytic activities. When cultivated on either malt extract (ME) liquid or wood-LNAS medium, all three isolates of *F. pinicola* produced extracellular acidic peptidase activities with increasing levels of activity over the cultivation period of 10 weeks (Fig. 2). In general, higher proteolytic activities were observed on ME medium than on wood-LNAS medium. On wood-LNAS medium, the highest peaks of activities were detected on cultivation week 8 (for *F. pinicola* FBCC1468 and FBCC1243) (Fig. 2a). On ME medium, isolates *F. pinicola* FBCC1181 and FBCC1243 in turn demonstrated more similar production patterns of acidic peptidase activity (Fig. 2b).

Extracellular chitinase activities from all three isolates were estimated from the culture filtrates on both media (Fig. 3). On wood-LNAS medium, low levels of chitinase production were already observed, with activities peaking on the first week of cultivation for all isolates, and a later activity peak appeared on week 4 for the *F. pinicola* isolate FBCC1243 (Fig. 3a). On ME medium, *F. pinicola* isolates FBCC1181 and FBCC1468 maintained low levels of chitinase activity until week 6 of cultivation, while the *F. pinicola* isolate FBCC1243 demonstrated an increasing activity accumulation pattern until cultivation week 7, which then suddenly dropped within 2 weeks (cultivation weeks 7 to 9) to zero levels (Fig. 3b).

Ergosterol content and fungal growth. Ergosterol analysis demonstrated the presence of active mycelial growth for all three isolates on both media during 12 weeks

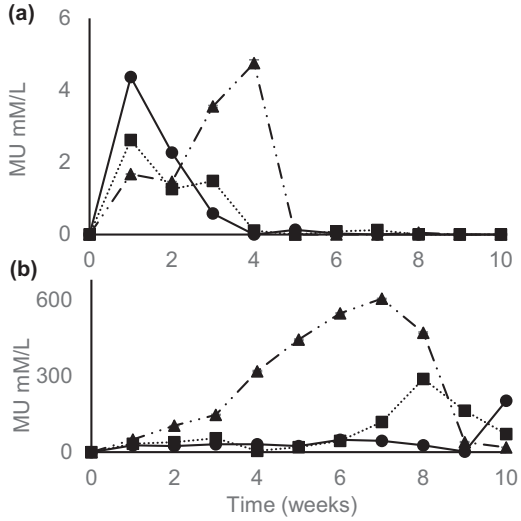

FIG 3 Extracellular chitinase activity of *F. pinicola* isolates on wood-LNAS (a) and ME (b) media during 10 weeks of cultivation. Isolates shown are FBCC1181 (dotted line, closed square), FBCC1243 (long dash-dot, closed triangle), and FBCC1468 (continuous line, closed circle). Chitinase activity is represented as the amount of 4-methylumbelliferone (MU) released per liter of culture filtrate. Bars indicate SE (n = 5, biological replicates). In some cases, the error bar is not visible due to small deviation values.

of cultivation (Fig. 4, and see Table S1 in the supplemental material). On wood-LNAS medium, fungal growth was slower but continuous, according to ergosterol concentration in the extracted fungus-colonized wood (Fig. 4a). However, after rapid mycelial ergosterol accumulation on ME medium until cultivation week 6, the ergosterol content declined by week 8 but showed an increased amount of ergosterol by the end of cultivation (on week 12) (Fig. 4b).

Oxalic acid and extracellular pH. On both cultivation media (ME broth and wood-LNAS), the three *F. pinicola* isolates accumulated substantial concentrations of oxalic acid over the cultivation period, resulting in a marked decrease in the culture

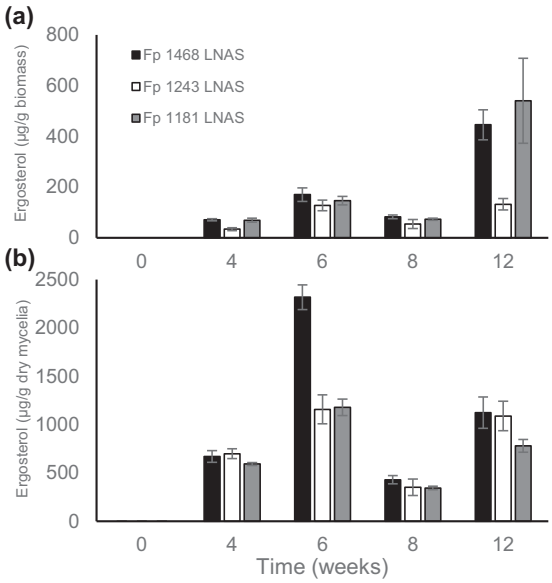

FIG 4 Ergosterol content of the *F. pinicola* isolates on wood-LNAS (a) and ME (b) media during 12 weeks of cultivation. Ergosterol content is estimated per gram (dry weight) of fungus-colonized wood (wood-LNAS) and dry weight fungal mycelia (ME). Bars indicate the SE (n = 3, biological replicates). In some cases, the error bar is not visible due to small deviation values. Fp 1468, *F. pinicola* FBCC1468; Fp 1243, *F. pinicola* FBCC1243; Fp 1181, *F. pinicola* FBCC1181.

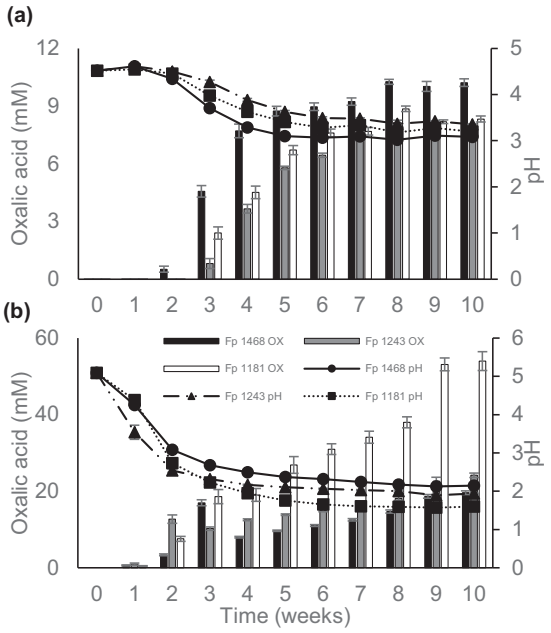

FIG 5 Accumulation of secreted oxalic acid and decrease in culture filtrate pH during cultivations of the three *F. pinicola* isolates on wood-LNAS (a) and ME (b) media over the 10-week cultivation period. Oxalic acid concentrations are represented as columns, and pH values are indicated as line graphs. The key identifies oxalic acid production (OX) and pH for both media. Bars indicate SE ($n = 5$, biological replicates). In some cases, the error bar is not visible due to small deviation values.

fluid pH (Fig. 5). Even on the buffered wood-LNAS medium (pH 4.5), increasing acidity dropped the pH to 3.0 to 3.5 in 5 weeks of cultivation (Fig. 5a). *F. pinicola* FBCC1468 showed the highest accumulation of 10 mM oxalic acid on cultivation week 8. However, even more intense accumulation of oxalic acid was observed with all three isolates when they were cultivated on ME medium, with oxalic acid concentrations reaching over 50 mM in cultures of *F. pinicola* FBCC1181 on week 9 (Fig. 5b). Accordingly, the culture fluid pH values dropped dramatically on ME medium with a decrease in the pH from the initial value of pH 5.0 to pH 1.5 to 2.0 already during the two first weeks of cultivation.

Fe^{3+}-reducing activity. Extracellular Fe^{3+}-reducing activity produced by the three *F. pinicola* isolates cultivated on ME and wood-LNAS media was analyzed from the culture fluid samples and from ethyl acetate-extracted concentrates at time points 0, 2, 8, and 12 weeks of cultivation (Fig. 6). Trace amounts of Fe^{3+}-reducing activity were detected on both media (ME liquid and wood-LNAS) at the start of the cultivations (0 week). On both cultivation media, all isolates released variable amounts of extracellular Fe^{3+}-reducing activity over the cultivation period, with no distinct temporal tendency. However, the ethyl acetate extracts demonstrated noticeable Fe^{3+}-reducing activities, suggesting the constitutive presence of Fe^{3+}-reducing metabolites over the cultivation period in wood-LNAS (Fig. 6a) and ME (Fig. 6b) media. Variability in the Fe^{3+} reduction activities may be a consequence of an accumulation of fungus-produced oxalic acid in the cultures, which was tested by the addition of reference oxalic acid (in Milli-Q water) in various concentrations (0 to 50 mM) to the ethyl acetate extract of isolate *F. pinicola* FBCC1468 from the 12-week cultures on ME broth (Fig. S1).

Fungal metabolites and Fe^{3+}-reducing activity. In order to examine aromatic extracellular metabolites produced by the three *F. pinicola* isolates on ME liquid and wood-LNAS media at the endpoint of the cultivations (on week 12), ethyl acetate extracts of culture fluid filtrates were further analyzed by reverse-phase high-performance liquid chromatography (HPLC). Multiple potential aromatic compounds were separated from cultures of the three isolates, as indicated by the set of peaks detected at 280 nm of absorbance (Fig. 7 and Table 1). HPLC chromatograms recorded

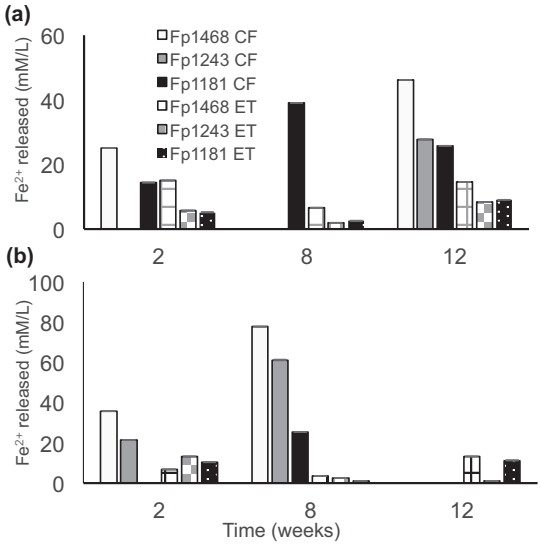

FIG 6 Fe^{2+} generation by the culture filtrate (CF) and ethyl acetate-extracted culture filtrate (ET) obtained from wood-LNAS (a) and ME (b) media. Activities for time points of 2, 8, and 12 weeks of cultivation are presented for all three *F. pinicola* isolates and are normalized against Fe^{2+} generation detected in the uninoculated sterile medium (control samples derived on day 0 of cultivations). Fe^{2+} generated is represented per liter of culture filtrate for both CF and ET. Bars indicate SE ($n = 3$, biological replicates). In some cases, the error bar is not visible due to small deviation values.

at 260 nm (data not shown) indicated minor differences (mainly in the peak absorbance intensities) in comparison to the peak profiles recorded at 280 nm.

All three isolates demonstrated similar profiles of extracellular aromatic compounds on both media, with apparently a more versatile set of aromatic compounds secreted on ME medium than was observed on wood-LNAS medium (Table 1 and Fig. 7, S2, and S3). On ME medium, *F. pinicola* isolates accumulated aromatic compounds (in 12 weeks) which were detected at 280 nm absorbance at retention times (RT) of 1.440, 2.031 to 2.042, 4.890, 10.012, and 11.221 min, and Fe^{3+}-reducing activity was detected in three of these peak fractions (RT peaks 1.440, 2.031 to 2.042, and 10.012 min) (Table 1 and Fig. 7). On wood-LNAS medium, aromatic compounds (as detected at 280 nm of absorbance) accumulated at RTs of 1.442, 2.799, and 10.125 min, with Fe^{3+}-reducing activity detected for two of the fractions (RT peaks 1.442 and 10.125 min) (Table 1 and Fig. 7). A similar pattern in the generation of aromatic compounds was observed with all three *F. pinicola* isolates on both media studied, with the same HPLC-isolated compound fractions demonstrating Fe^{3+}-reducing activity shared between the isolates (Table 1 and Fig. 6, S3, and S4); this indicates on a fungal species level a constitutive specificity for the production of bioactive metabolites.

DISCUSSION

The biochemical mechanisms involved in the biodegradation of lignocelluloses and wood by the brown rot fungi is understood to be acquired as an alternative to the enzymatically and genetically more-expanded apparatus employed by the white rot fungi (5–7, 18). Brown rot fungi are still efficient decomposers of lignocellulose, expressing strong oxidative biochemistry (13, 14, 24) and CAZyme hydrolytic enzymes active on cellulose, hemicellulose, and pectin (1, 5, 6, 18–20, 23, 26). The mechanisms, however, are not yet fully understood, and variations of the biochemical processes and enzyme expression are expected to have evolved in the systematic lineages of Agaricomycetes Polyporales brown rot fungi. Emerging evidence relies on a nonenzymatic free radical system generated via Fenton chemistry to act on the lignocellulose structures leading to the modification of hemicellulose linkages and lignin units, thus facilitating the binding and infiltration of fungus-secreted CAZymes acting on cellulose chains and hemicellulose polysaccharides (7, 9, 19).

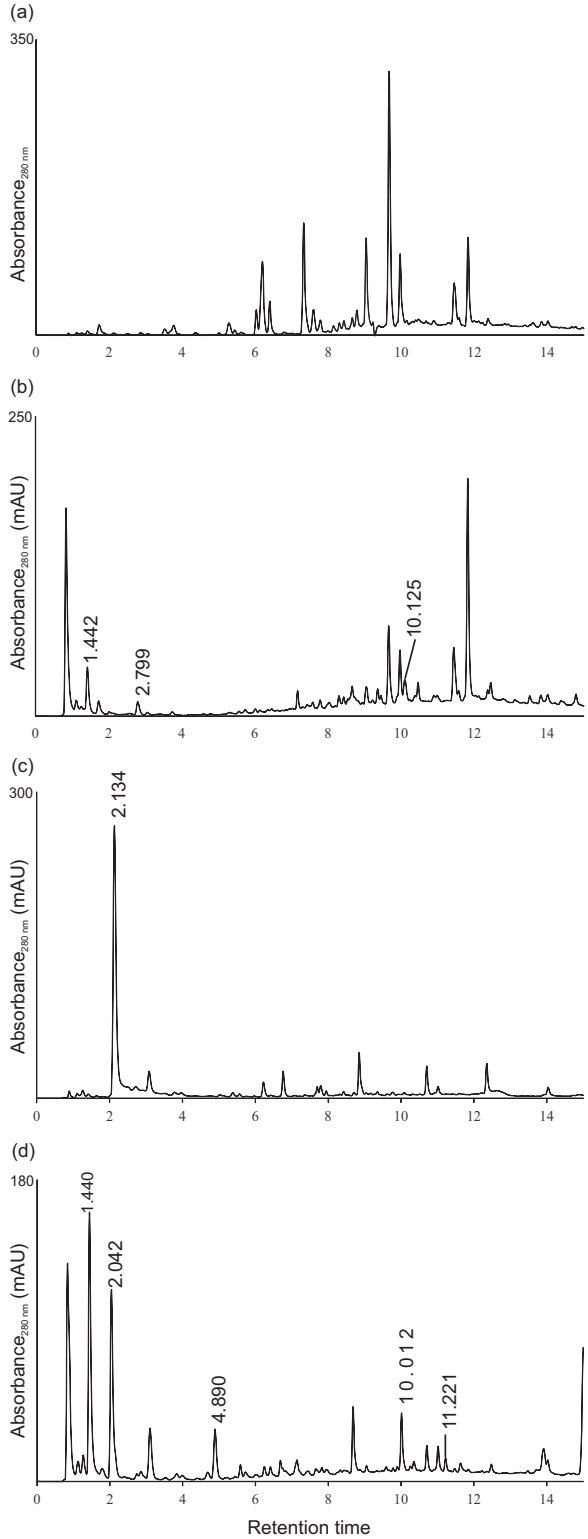

FIG 7 Aromatic compound profiles in cultures of the *F. pinicola* isolate FBCC1468 cultivated on wood-LNAS and ME media. HPLC chromatograms of ethyl acetate-extracted culture filtrates were derived at time points of 0, initial medium-dissolved compound profile of wood-LNAS substrate at the beginning of cultivation (a); after 12 weeks of cultivation on wood-LNAS (b); 0, initial medium-dissolved compound profile of ME substrate at the beginning of cultivation (c); and after 12 weeks of cultivation on ME medium (d). Aromatic compound peaks predicted to be generated as either secretion products or by bioconversion of medium-existing aromatic and organic molecules are indicated with their retention times. For peaks with Fe^{3+}-reducing activities, see Table 1.

TABLE 1 Aromatic compound peaks analyzed and collected by HPLC from the ethyl acetate-extracted culture filtrate samples of the three isolates of *F. pinicola* cultivated on wood-LNAS and ME media

	RT (min) at A_{280}[a]			
	Aromatic compound peaks in cultures		Fe^{3+}-reducing peaks	
Fungal isolate	wood-LNAS	ME	wood-LNAS	ME
FBCC1181	**1.418**, 2.799, **10.119**	**1.440**, **2.031**, 2.377, 4.855, **9.999**	1.418, 10.119	1.440, 2.031, 9.999
FBCC1243	**1.420**, 2.796, **10.127**	1.822, **2.031**, **9.999**	1.420, 10.127	2.031, 9.999
FBCC1468	**1.442**, 2.799, **10.125**	**1.440**, **2.042**, 4.890, **10.012**, 11.221	1.442, 10.125	1.440, 2.042, 10.012

[a]Fe^{3+}-reducing activity of released peaks were determined using ferrozine assay. RTs in bold type indicate those with Fe^{3+}-reducing activity (to the right).

According to structural localization study on decayed wood, it has been concluded that brown rot fungi depolymerize hemicellulose prior to cellulose and degrade all polysaccharides upon the decay process (19–23, 27). This general order of polysaccharide decomposition is supported by our study; all three *F. pinicola* isolates demonstrated extracellular hemicellulose-targeted xylanase activity on the spruce wood-containing medium (wood-LNAS medium). Xylanase activity was produced early on during the first week of cultivation, demonstrating a repeating pattern of enzyme activity during the cultivation period of 10 weeks. Surprisingly, *F. pinicola* FBCC1243 expanded high xylanase activity during the same cultivation period. A repeating pattern was also presented with cellulose-attacking endo-β-1,4-glucanase activity, with some time-dependent differences between the three *F. pinicola* isolates. Likewise, a time-dependent pattern in endo-β-1,4-glucanase activity has been observed for another Polyporales brown rot species, *Postia (Rhodonia) placenta,* on solid wood (28).

In the genomes of *P. placenta* and *F. pinicola*, multiple genes encoding CAZyme glycoside hydrolase (GH) classes responsible for xylanase and endo-β-1,4-glucanase activities are identified (6, 18), thus suggesting the expression of different genes producing divergent xylanases and endoglucanase enzymes in the course of fungal hyphal growth and developing decay of wood. Previous studies on the production of cellulose-attacking endo-β-1,4-glucanase activity by the evolutionarily distantly related brown rot species *Gloeophyllum trabeum* proposed that the enzymes are expressed constitutively and are thereby not subjected to accumulating glucose-influenced catabolic repression during lignocellulose decay (29, 30). Constitutive production of endoglucanase activity is also supported by our results on the three isolates of *F. pinicola*, thus pinpointing the importance of these CAZyme activities in the brown rot decay of wood.

Contrary to the cellulolytic endoglucanase activity, brown rot fungi are known to show low β-glucosidase activity (4). This may be due to the β-glucosidase enzyme redundancy and differences in substrate specificities and cellular localization (5–7, 18), but it might also be due to catabolic repression by glucose, as an end product of the activity, and the potential accumulation of glucose in the culture fluid (19). Accordingly, the three *F. pinicola* isolates secreted overall low levels of β-glucosidase activity compared to the other CAZyme activities analyzed, with a slightly fluctuating activity pattern observed during the long course of the cultivation period. As observed for endoglucanase activity, this pattern can be attributed to differential regulation of multiple β-glucosidase-encoding genes being expressed and subsequent isoenzymes secreted by the fungal isolates.

Wood-decaying fungi secrete proteases and chitinases to acquire nitrogen and to regenerate the fungal mycelium during growth (31, 32). The three isolates of *F. pinicola* presented a gradual increase in extracellular acidic peptidase activity over the cultivation period, suggesting for a need for available organic nitrogen sources as the mycelia were decomposing their lignocellulose substrates, particularly on the semisolid wood-LNAS medium. Proteolytic enzymes from *F. pinicola* have been reported to be active at acidic pH (31), which is in accordance with our results on increasing acidic (pH 4.0 in assay) peptidase activity detected for the three isolates during the cultivation period. Various degrees of extracellular chitinase activity were also observed for the *F. pinicola* isolates, suggesting intimate regulation of chitin biosynthesis and degradation, as has been proposed for filamentous fungi (32).

Accumulation of oxalic acid is a distinguishable characteristic of brown rot fungi, and their ability to produce extracellular oxalic acid exceeds that of the evolutionarily closely related white rot fungi, increasing in concentration by up to several 10-fold (25, 33). We observed an increase in the accumulation of oxalic acid during growth of the three *F. pinicola* isolates, with even higher concentrations (over 50 mM) on the richer ME medium. This may primarily be a yield effect and consequence of the stronger mycelial growth on ME medium, as was reflected by the substantially greater content of ergosterol determined in the mycelia from ME cultivations than from the wood-LNAS cultures. Although significant secretion of oxalic acid is common with *F. pinicola* (17, 25), differences in the quantities of oxalic acid production were observed between the three isolates in our study. Isolate *F. pinicola* FBCC1468 presented oxalic acid secretion more readily on the spruce wood-LNAS medium, while the isolate *F. pinicola* FBCC1181 was the best producer of oxalic acid on the rich ME medium. Despite the differences in oxalic acid production, final pH values of the cultures decreased to similar low levels by all three *F. pinicola* isolates (around pH 3 on wood-LNAS medium and around pH 2 on ME medium). Increasing extracellular acidity may, in turn, affect CAZyme activities attacking hemicelluloses and cellulose, as well as protein-degrading enzymes (4, 34).

Oxalic acid and low pH have been implicated in Fenton chemistry-based free radical generation by brown rot fungi (9, 16, 17, 33). *In vitro* experiments have demonstrated that oxalic acid as soluble oxalate anions may easily chelate metal cations, like ferric iron from wood, in the low-pH environment in the vicinity of fungal hyphae within the wood tracheid lumen area, thus preventing the reduction of iron species and subsequent generation of hydroxyl radicals via a Fenton reaction (16, 17, 24). In this study, we observed that the three *F. pinicola* isolates demonstrated Fe^{3+}-reducing activities on both substrate media and over the long cultivation period. The Fe^{3+}-reducing extracellular activity was found to be present during the long cultivation period, as is shown by the ethyl acetate extracts, suggesting for a constant presence or continuous secretion of extracellular metabolites with Fe^{3+}-reducing potential. The Fe^{3+}-reducing activity observed in the culture fluid extracts may also suggest of inhibitory effect of oxalic acid on Fe^{3+} reduction. This conclusion was further supported by the observed decline in Fe^{3+}-reducing activity of culture fluid extracts of isolate *F. pinicola* FBCC1468 by the addition of increasing molar concentrations of oxalic acid. Unlike in a recent study distinguishing fungal hypha-generated biochemical gradients and decay phases of brown rot on wood by *P. placenta* (26, 44), our goal here was to initially analyze the enzymatic and nonenzymatic players in the lignocellulose-degradative system of another Polyporales fungus, the species *F. pinicola*. Our next aim was to identify these processes on solid-wood cultivations in order to aid in a comparison of the proposed several-stage and "staggered" decay mechanism of *P. placenta* (26) to the decay events and physiology of *F. pinicola*, for order-level functional and comparative meta-analyses, and in general to achieve more knowledge on the physiology of brown rot Polyporales fungi.

Basidiomycota hypha-secreted metabolites have been reported to possess Fe^{3+}-reducing activity while growing on wood and lignocellulose-supplemented medium or on soil extracts, and these biocompounds have been as well implicated to play a role in Fenton chemistry (3, 5, 35). In this study, we managed to identify a number of aromatic compound fractions secreted by the three isolates of *F. pinicola*, with a few accumulating compounds showing Fe^{3+}-reducing activity. A number of these potential iron-reducing compounds were produced by all three isolates, thus suggesting both (i) a constitutive biosynthetic pathway for the production of specific bioactive secondary metabolites (as was observed on ME medium), and (ii) similarities in the bioconversion of spruce wood-derived soluble aromatic compounds (as was observed on wood-LNAS medium). Thus, species-specific Fenton chemistry and the wood decay mechanism may be tentatively formulated for *F. pinicola*. Identification of these extracellular bioactive compounds is our next step in order to elucidate which roles they may play in Fenton chemistry and brown rot decay generated by the fungus.

Our study confirmed that the three forest wild-type isolates of *F. pinicola* demonstrated extracellular enzyme activity to degrade lignocellulose polysaccharides and proteolytic substrates, with the potential for oxidative decay via Fenton chemistry, which was supported by the strong secretion of oxalic acid and production of aromatic compounds. A few of the compounds were able to cause reduction of ferric iron species to ferrous ions. The production of the extracellular bioactive compounds with Fe^{3+}-reducing potential together with CAZyme activity suggests for a complex and fine-tuned mechanism that can be genetically and biochemically regulated by the fungus. Further analyses on the fungal proteome and functional genomics on wood and characterization of the reducing-activity metabolites are necessary steps to uncover the brown rot decay system of *F. pinicola*.

MATERIALS AND METHODS

Fungal isolates. Three wild-type isolates of the species *Fomitopsis pinicola* (FBCC1181, FBCC1468, and FBCC1243) were obtained as living hyphal stored cultures from the University of Helsinki Microbiology-Fungal Biotechnology Culture Collection (HAMBI-FBCC). The identity and taxonomy of the isolates were confirmed by internal transcribed spacer PCR (ITS-PCR) and BLASTN analyses on the ITS1-5.8S-ITS2 contig sequences (25), resulting in a BLAST E value of 0.0 and 99 to 100% sequence identities with other isolates of *F. pinicola*. The fungal isolates were maintained on 2% (wt/vol) malt extract (Biokar Diagnostics, France) agar plates at 25°C and in the dark.

Culture conditions and media. To determine enzyme activity and metabolite production, the isolates were cultivated as previously described (36). Culture media adopted for the enzyme activities and metabolite production were 1% (wt/vol) malt extract liquid (pH 5.5; Biokar Diagnostics, France) (ME broth), and low-nitrogen asparagine-succinate medium (pH 4.5) containing 1% (dry wt/vol) of spruce (*Picea abies*) sawdust wood (wood-LNAS medium). The carbon sources in the media were either dissolved carbohydrates (ME) or ground spruce wood (wood-LNAS). The media were autoclaved (121°C, 15 min) before use.

Each *F. pinicola* isolate was precultivated in 100 ml of ME broth for 1 week at 25°C in the dark as stationary cultures (nonagitated). The mycelia were homogenized in the broth using a sterilized steel container and Waring blender. Aliquots of 2 ml of the mycelial homogenate were adopted to inoculate 250-ml flasks containing 100 ml of autoclaved ME broth or wood-LNAS medium and closed with sterilized spongy stoppers. Each fungal isolate was cultivated in five parallel flasks on both media and incubated at 25°C in the dark as stationary cultures over periods of 12 weeks of cultivation. Sample volumes of 1.5 ml for enzyme activity measurements were aseptically taken at time points 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 weeks. Control medium flasks without fungi were incubated similarly. Metabolite profiles and analysis of Fe^{3+} reduction were also followed up to 12 weeks of cultivation.

Enzyme activities. Lignocellulose-attacking enzyme activities produced in five parallel culture flasks of each isolate on the wood-LNAS medium were measured by using 96-well plate assays and an Infinite M200 microplate reader spectrophotometer (Tecan, Switzerland). The reaction volume was 250 μl, and three parallel reactions (technical replicates) were measured for each sample from each fungal culture flask. Cellulolytic endo-β-1,4-glucanase (EC 3.2.1.4) (endoglucanase) and β-glucosidase (EC 3.2.1.21) activity reactions were performed in 50 mM sodium citrate buffer (pH 5) at 45°C (37). The annotated genome of *F. pinicola* indicates the absence of genes encoding CAZyme family GH6 or GH7 cellobiohydrolase (CBH, exocellulase) (6, 43). Thus, specific CBH activity was not assayed in this study. Endo-β-1,4-glucanase activity was determined with 1% (wt/vol) hydroxyethyl cellulose (Sigma-Aldrich) as the substrate (37). Endo-β-1,4-xylanase (EC 3.2.1.8) activity was assayed with birch xylan (Sigma-Aldrich) as the substrate, and reducing sugars were measured with dinitrosalicylic acid (DNS) at 540 nm (38). β-Glucosidase activity was assayed by quantifying the amount of *p*-nitrophenol released from 1 mM 4-nitrophenyl-β-D-glucopyranoside (Sigma-Aldrich) at 400 nm (37). Solutions of various concentrations of *p*-nitrophenol were prepared as references for the quantitation of product formation in the enzyme kinetic assays. Trials for the laccase activity assay using 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS) as the substrate (25, 36) were performed for all three isolates on both media.

Extracellular proteolytic activity was measured using a modified version of the previously adopted method (34). The conjugate fluorescein isothiocyanate coupled with bovine serum albumin (FITC-BSA; Sigma-Aldrich) was used as the substrate. Briefly, 100 μl of the sample was mixed with an equal volume of buffer (10 mM citrate-HCl [pH 4.0]) and 10 μl of FITC-BSA (1 mg $\cdot$ ml^{-1} in citrate buffer), and the mixture was incubated at 37°C for 4 h. The reaction was terminated by adding 100 μl of 10% (wt/vol) trichloroacetic acid. After 1 h of incubation at room temperature in the dark, the sample was centrifuged at 16,000 $\times$ *g* for 5 min at room temperature. Then, 5 μl of the supernatant was mixed with 200 μl of 0.4 M boric acid-NaOH buffer (pH 8.7) in a 96-well plate. Fluorescence was measured using a spectrofluorometer (Victor; PerkinElmer) with excitation set to 495 nm and emission set to 520 nm. One fluorescence unit corresponds to 6.4 ng $\cdot$ ml^{-1} pepsin (Sigma-Aldrich) incubated in the citrate-HCl buffer (pH 4.0) under the same conditions at 37°C for 4 h.

Extracellular chitinase activity was estimated using a method adopted for fungi (39). 4-Methylumbelliferone (MU)–*N*-acetyl-β-D-glucosaminide (Sigma-Aldrich) was used as the substrate in 96-well plates. The release of the product, 4-methylumbelliferone, was measured using spectrofluorometer (Victor; PerkinElmer) at an excitation wavelength of 360 nm and emission at 450 nm. Calibration solutions

of 4-methylumbelliferone (Sigma-Aldrich) were prepared for product quantitation and enzyme activity calculations.

Oxalic acid analysis. Oxalic acid was chromatographically analyzed with Agilent 1290 Infinity binary LC 171 system (Agilent Technologies) coupled with a guard cartridge (Agilent Technologies) and Luna C_{18} column (150 mm by 4.6 mm, 3-μm particle size; Phenomenex). The column temperature was 40°C, and separation was conducted at a flow rate of 0.950 ml · min^{-1} under isocratic conditions by using an eluent mixture consisting of 95% (vol/vol) of 0.3% H_3PO_4 and 5% (vol/vol) acetonitrile (40). Samples were filtered using 0.2-μm-pore-size membrane filters (Chromacol RC; Thermo Scientific) before injection (2 μl). Chromatographic separation was recorded with Agilent diode-array detector, and the elution of oxalic acid was followed by the detection of absorbance at 210 nm and reference at 450 nm. Various concentrations of high-purity-grade oxalic acid (Sigma-Aldrich) were adopted to quantitate oxalic acid in the samples using an external standard method.

Ergosterol analysis. Total ergosterol was used to determine fungal growth and production of mycelial biomass in the flask cultures. Ergosterol was extracted from wood-LNAS and ME liquid cultures by freezing the wood meal and mycelium with liquid N_2 and then homogenizing with IKA 10 Analytical mill, or using mortar and pestle, respectively, at cultivation time points of 0, 4, 6, 8, and 12 weeks for all isolates. Ergosterol was extracted using established methods (41, 42), using three technical subsamples of 100 to 200 mg (raw weight) from each fungal replicate culture for all isolates, and measured by Agilent 1100 HPLC (Agilent Technologies), using previously described elution parameters and separating column with the detection of absorbance at 282 nm (41). A set of ergosterol (Sigma-Aldrich) concentrations were used as a reference.

Fe^{3+} reduction assay. Fe^{3+}-reducing activities were analyzed using a modified method of a ferrozine assay (35). A 50-μl aliquot of the sample was mixed with 1.0 ml of 0.1 M acetate buffer (pH 4.5), 25 μl of freshly prepared 1.0 mM $FeCl_3$, and 20 μl of 1% (wt/vol) aqueous ferrozine (Sigma-Aldrich). The reaction mixture was incubated for 5 min in the dark at room temperature. Fe^{3+} reduction was assayed spectrophotometrically at 562 nm. A standard curve was constructed using various concentrations of $FeSO_4$ (0.0 to 3.0 mM).

Extracellular metabolite profiling. Extracellular metabolites were extracted from the culture fluids by ethyl acetate extraction (35). Equal volumes of culture fluid filtrates obtained from the parallel culture flasks and ethyl acetate were mixed and vortexed, with the addition of 1 ml of 1 M HCl. The ethyl acetate phase was recovered and dried under a stream of N_2. The dried ethyl acetate phase was dissolved in methanol and analyzed by using the Agilent 1290 Infinity binary LC 171 system (Agilent Technologies) coupled with a guard cartridge and Kinetex C_{18} column (150 by 2.1 mm, 2.6-μm particle size; Phenomenex). A stepwise elution gradient consisting of 0.1% (vol/vol) formic acid in HPLC-grade water (solvent A) and 100% acetonitrile (solvent B) was applied at a flow rate of 0.36 ml/min, using a stepwise gradient elution (initial hold, 3 min with 95% solvent A and 5% of solvent B, followed by a linear increase in 21 min to 70% of solvent B, then in 6 min to 95% of solvent B, kept stable for further 3 min, and returning to the initial condition of 5% solvent B within 5 min). Elution was recorded using the Agilent diode-array detector, and chromatograms were processed at 210, 260, and 280 nm wavelengths with peak-actuated spectral scanning from 200 to 400 nm. Eluted fractions of fungus-produced organic compounds were collected based on their elution time and peak height and dried under N_2. Medium compounds were eliminated from the HPLC analyses according to comparison and subtraction from the fungal culture profiles. The concentrated fractions were further analyzed for their potential for Fe^{3+}-reducing activity.

Accession number(s). The ITS1-5.8S-ITS2 sequences of the isolates were deposited in the ENA nucleotide sequence data bank (http://www.ebi.ac.uk/ena) under accession numbers LT844580 (*F. pinicola* FBCC1181), LT906565 (*F. pinicola* FBCC1243), and LT906564 (*F. pinicola* FBCC1468).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02662-17.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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REFERENCES

1. Hori C, Gaskell J, Igarashi K, Samejima M, Hibbett D, Henrissat B, Cullen D. 2013. Genomewide analysis of polysaccharides degrading enzymes in 11 white- and brown-rot Polyporales provides insight into mechanisms of wood decay. Mycologia 105:1412–1427. https://doi.org/10.3852/13-072.
2. Binder M, Justo A, Riley R, Salamov A, Lopez-Giraldez F, Sjökqvist E, Copeland A, Foster B, Sun H, Larsson E, Larsson K-H, Townsend J, Grigoriev I, Hibbett DS. 2013. Phylogenetic and phylogenomic overview of the Polyporales. Mycologia 105:1350–1373. https://doi.org/10.3852/13-003.
3. Eriksson KEL, Blanchette RA, Ander P. 1990. Microbial and enzymatic degradation of wood and wood components. Springer, Berlin, Germany.

4. Baldrian P, Valaskova V. 2008. Degradation of cellulose by basidiomycetous fungi. FEMS Microbiol Rev 32:501–521. https://doi.org/10.1111/j.1574-6976.2008.00106.x.
5. Eastwood DC, Floudas D, Binder M, Majcherczyk A, Schneider P, Aerts A, Asiegbu FO, Baker SE, Barry K, Bendiksby M, Blumentritt M, Coutinho PM, Cullen D, de Vries RP, Gathman A, Goodell B, Henrissat B, Ihrmark K, Kauserud H, Kohler A, LaButti K, Lapidus A, Lavin JL, Lee YH, Lindquist E, Lilly W, Lucas S, Morin E, Murat C, Oguiza JA, Park J, Pisabarro AG, Riley R, Rosling A, Salamov A, Schmidt O, Schmutz J, Skrede I, Stenlid J, Wiebenga A, Xie XF, Kües U, Hibbett DS, Hoffmeister D, Hogberg N, Martin F, Grigoriev IV, Watkinson SC. 2011. The plant cell wall-decomposing machinery underlies the functional diversity of forest fungi. Science 333:762–765. https://doi.org/10.1126/science.1205411.
6. Floudas D, Binder M, Riley R, Barry K, Blanchette RA, Henrissat B, Martinez AT, Otillar R, Spatafora JW, Yadav JS, Aerts A, Benoit I, Boyd A, Carlson A, Copeland A, Coutinho PM, de Vries RP, Ferreira P, Findley K, Foster B, Gaskell J, Glotzer D, Gorecki P, Heitman J, Hesse C, Hori C, Igarashi K, Jurgens JA, Kallen N, Kersten P, Kohler A, Kues U, Kumar TK, Kuo A, LaButti K, Larrondo LF, Lindquist E, Ling A, Lombard V, Lucas S, Lundell T, Martin R, McLaughlin DJ, Morgenstern I, Morin E, Murat C, Nagy LG, Nolan M, Ohm RA, Patyshakuliyeva A, et al. 2012. The Paleozoic origin of enzymatic lignin decomposition reconstructed from 31 fungal genomes. Science 336:1715–1719. https://doi.org/10.1126/science.1221748.
7. Lundell TK, Mäkelä MR, de Vries RP, Hildén KS. 2014. Genomics, lifestyles and future prospects of wood-decay and litter-decomposing Basidiomycota. Adv Bot Res 70:29–370.
8. Schwarze FWMR. 2007. Wood decay under the microscope. Fungal Biol Rev 21:133–170. https://doi.org/10.1016/j.fbr.2007.09.001.
9. Arantes V, Jellison J, Goodell B. 2012. Peculiarities of brown rot fungi and biochemical Fenton reaction with regard to their potential as a model for bioprocessing biomass. Appl Microbiol Biotechnol 94:323–338. https://doi.org/10.1007/s00253-012-3954-y.
10. Högberg N, Holdenrieder O, Stenlid J. 1999. Population structure of the wood decay fungus *Fomitopsis pinicola*. Heredity (Edinb) 83:354–360. https://doi.org/10.1038/sj.hdy.6885970.
11. Hammel KE, Kapich AN, Jensen KA, Jr, Ryan ZC. 2002. Reactive oxygen species as agents of wood decay by fungi. Enzyme Microb Technol 30:445–453. https://doi.org/10.1016/S0141-0229(02)00011-X.
12. Koenigs JW. 1974. Hydrogen peroxide and iron: a proposed system for decomposition of wood by brown rot basidiomycetes. Wood Fiber 6:66–79.
13. Kerem Z, Jensen KA, Hammel KE. 1999. Biodegradative mechanism of the brown rot basidiomycete *Gloeophyllum trabeum*: evidence for an extracellular hydroquinone-driven Fenton reaction. FEBS Lett 446:49–54. https://doi.org/10.1016/S0014-5793(99)00180-5.
14. Jensen KA, Jr, Houtman CJ, Ryan ZC, Hammel KE. 2001. Pathways for extracellular Fenton chemistry in the brown rot basidiomycete *Gloeophyllum trabeum*. Appl Environ Microbiol 67:2705–2711. https://doi.org/10.1128/AEM.67.6.2705-2711.2001.
15. Hyde SM, Wood PM. 1997. A mechanism for production of hydroxyl radicals by the brown rot fungus *Coniophora puteana*: Fe(III) reduction by the cellobiose dehydrogenase and Fe(II) oxidation at a distance from the hyphae. Microbiology 143:259–266. https://doi.org/10.1099/00221287-143-1-259.
16. Arantes V, Qian Y, Milagres AMF, Jellison J, Goodell B. 2009. Effect of pH and oxalic acid on the reduction of Fe^{3+} by a biomimetic chelator and on Fe^{3+} desorption/adsorption onto wood: implications for brown rot decay. Int Biodeterior Biodegradation 63:478–483. https://doi.org/10.1016/j.ibiod.2009.01.004.
17. Schilling JS, Jellison J. 2005. Oxalate regulation by two brown rot fungi decaying oxalate-amended and non-amended wood. Holzforschung 59:681–688.
18. Martinez D, Challacombe J, Morgenstern I, Hibbett D, Schmoll M, Kubicek CP, Ferreira P, Ruiz-Duenas FJ, Martinez AT, Kersten P, Hammel KE, Vanden Wymelenberg A, Gaskell J, Lindquist E, Sabat G, Bondurant SS, Larrondo LF, Canessa P, Vicuna R, Yadav J, Doddapaneni H, Subramanian V, Pisabarro AG, Lavin JL, Oguiza JA, Master E, Henrissat B, Coutinho PM, Harris P, Magnuson JK, Baker SE, Bruno K, Kenealy W, Hoegger PJ, Kues U, Ramaiya P, Lucas S, Salamov A, Shapiro H, Tu H, Chee CL, Misra M, Xie G, Teter S, Yaver D, James T, Mokrejs M, Pospisek M, Grigoriev IV, Brettin T, et al. 2009. Genome, transcriptome, and secretome analysis of wood decay fungus *Postia placenta* supports unique mechanisms of lignocellulose conversion. Proc Natl Acad Sci U S A 106:1954–1959. https://doi.org/10.1073/pnas.0809575106.
19. Valáskova V, Baldrian P. 2006. Degradation of cellulose and hemicelluloses by the brown rot fungus *Piptoporus betulinus*—production of extracellular enzymes and characterization of the major cellulases. Microbiology 152:3613–3622. https://doi.org/10.1099/mic.0.29149-0.
20. Milagres AMF, Sales RM. 2001. Evaluating the basidiomycetes *Poria medulapanis* and *Wolfiporia cocos* for xylanase production. Enzyme Microb Technol 28:522–526. https://doi.org/10.1016/S0141-0229(00)00364-1.
21. Highley TL. 1987. Effect of carbohydrate and nitrogen on hydrogen peroxide formation by wood decay fungi in solid medium. FEMS Microbiol Lett 48:373–377. https://doi.org/10.1111/j.1574-6968.1987.tb02627.x.
22. Highley TL, Illman BL. 1991. Progress in understanding how brown rot fungi degrade cellulose. Biodeterior Abstr 5:231–244.
23. Polizeli MLTM, Rizzatti ACS, Monti R, Terenzi HF, Jorge JA, Amorim DS. 2005. Xylanases from fungi: properties and industrial applications. Appl Microbiol Biotechnol 67:577–591. https://doi.org/10.1007/s00253-005-1904-7.
24. Goodell B, Jellison J, Liu J, Daniel G, Paszczynski A, Fekete F, Krishnamurthy S, Jun L, Xu G. 1997. Low molecular weight chelators and phenolic compounds isolated from wood decay fungi and their role in the fungal biodegradation of wood. J Biotechnol 53:133–162. https://doi.org/10.1016/S0168-1656(97)01681-7.
25. Mali T, Kuuskeri J, Shah F, Lundell TK. 2017. Interactions affect hyphal growth and enzyme profiles in combinations of coniferous wood-decaying fungi of Agaricomycetes. PLoS One 12:e0185171. https://doi.org/10.1371/journal.pone.0185171.
26. Zhang J, Presley GN, Hammel KE, Ryu J-S, Menke JR, Figueroa M, Hu D, Orr G, Schilling JS. 2016. Localizing gene regulation reveals a staggered wood decay mechanism for the brown rot fungus *Postia placenta*. Proc Natl Acad Sci U S A 27:10968–10973. https://doi.org/10.1073/pnas.1608454113.
27. Blanchette RA, Abad AR. 1988. Ultrastructural localization of hemicellulose in birch wood (*Betula papyfera*) decayed by brown and white rot fungi. Holzforschung 42:393–398. https://doi.org/10.1515/hfsg.1988.42.6.393.
28. Duncan SM, Schilling JS. 2010. Carbohydrate-hydrolyzing enzyme ratios during fungal degradation of woody and non-woody lignocellulose substrates. Enzyme Microb Technol 47:363–371. https://doi.org/10.1016/j.enzmictec.2010.07.007.
29. Cotoras M, Agosin E. 1992. Regulatory aspects of endoglucanase production by the brown rot fungus *Gloeophyllum trabeum*. Exp Mycol 16:253–260. https://doi.org/10.1016/0147-5975(92)90001-8.
30. Highley TL. 1973. Influence of carbon source on cellulase activity of white rot and brown rot fungi. Wood Fiber Sci 5:50–58.
31. Kudryavtseva OA, Dunaevsky YE, Kamzolkina OV, Belozersky MA. 2008. Fungal proteolytic enzymes: features of the extracellular proteases of xylotrophic basidiomycetes. Microbiology 77:643–653. https://doi.org/10.1134/S0026261708060015.
32. Langner T, Göhre V. 2016. Fungal chitinases: function, regulation, and potential roles in plant/pathogen interactions. Curr Genet 62:243–254. https://doi.org/10.1007/s00294-015-0530-x.
33. Mäkelä MR, Hildén K, Lundell TK. 2010. Oxalate decarboxylase: biotechnological update and prevalence of the enzyme in filamentous fungi. Appl Microbiol Biotechnol 87:801–814. https://doi.org/10.1007/s00253-010-2650-z.
34. Shah F, Rineau F, Canbäck B, Johansson T, Tunlid A. 2013. The molecular components of the extracellular protein-degradation pathways of the ectomycorrhizal fungus *Paxillus involutus*. New Phytol 200:875–887. https://doi.org/10.1111/nph.12425.
35. Shah F, Schwenk D, Nicolas C, Persson P, Hoffmeister D, Tunlid A. 2015. Involutin is a Fe^{3+} reductant secreted by the ectomycorrhizal fungus *Paxillus involutus* during Fenton-based decomposition of organic matter. Appl Environ Microbiol 81:8427–8433. https://doi.org/10.1128/AEM.02312-15.
36. Kuuskeri J, Mäkela MR, Isotalo J, Oksanen I, Lundell T. 2015. Lignocellulose-converting enzyme activity profiles correlate with molecular systematics and phylogeny grouping in the incoherent genus *Phlebia* (Polyporales, Basidiomycota). BMC Microbiol 15:217. https://doi.org/10.1186/s12866-015-0538.x.
37. Rytioja J, Hildén K, Hatakka A, Mäkelä MR. 2014. Transcriptional analysis of selected cellulose-acting enzymes encoding genes of the white rot fungus *Dichomitus squalens* on spruce wood and microcrystalline cellulose. Fungal Genet Biol 72:91–98. https://doi.org/10.1016/j.fgb.2013.12.008.
38. Bailey MJ, Biely P, Poutanen K. 1992. Interlaboratory testing of methods for assay of xylanase activity. J Biotechnol 23:257–270. https://doi.org/10.1016/0168-1656(92)90074-J.

39. Pritsch K, Courty PE, Churin J-L, Cloutier-Hurteau B, Ali MA, Damon C, Duchemin M, Egli S, Ernst J, Fraissinet-Tachet L, Kuhar F, Legname E, Marmeisse R, Müller A, Nikolova P, Peter M, Plassard C, Richard F, Schloter M, Selosse M-A, Franc A, Garbaye J. 2011. Optimized assay and storage conditions for enzyme activity profiling of ectomycorrhizae. Mycorrhiza 21:589–600. https://doi.org/10.1007/s00572-011-0364-4.
40. Mattila H, Kuuskeri J, Lundell T. 2017. Single-step, single-organism bioethanol production and bioconversion of lignocellulose waste materials by phlebioid fungal species. Bioresour Technol 225:254–261. https://doi.org/10.1016/j.biortech.2016.11.082.
41. Niemenmaa O, Galkin S, Hatakka A. 2008. Ergosterol contents of some wood-rotting basiodiomycete fungi grown in liquid and solid culture conditions. Int Biodeterior Biodegradation 62:125–134. https://doi.org/10.1016/j.ibiod.2007.12.009.
42. Frostegård A, Bååth E. 1996. The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. Biol Fertil Soils 22:59–65. https://doi.org/10.1007/BF00384433.
43. Ruiz-Dueñas FJ, Lundell T, Floudas D, Nagy LG, Barrasa JM, Hibbett DS, Martinez AT. 2013. Lignin-degrading peroxidases in Polyporales: and evolutionary survey based on 10 sequenced genomes. Mycologia 105:1428–1444. https://doi.org/10.3852/13-059.
44. Schilling JS, Duncan SM, Presley GN, Filley TR, Jurgens JA, Blanchette RA. 2013. Colocalizing incipient reactions in wood degraded by the brown rot fungus *Postia placenta*. Int Biodeterior Biodegradation 83:56–62. https://doi.org/10.1016/j.ibiod.2013.04.006.