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Hydrolytic and oxidative enzymes produced by white- and brown-rot fungi during *Eucalyptus grandis* decay in solid medium

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Abstract

Two species o brown-rot (BR) fungi and four of white-rot (WR) fungi were grown on *Eucalyptus grandis* wood chips under solid-state fermentation. BR fungi produced high levels of hydrolytic activities and no phenoloxidase activity, whereas WR fungi produced hydrolytic and ligninolytic enzymes, but low levels of hydrolytic activities in comparison with BR fungi. The capacity of WR fungi for wood degradation (determined as weight and component losses) seemed to be correlated with the levels of oxidative activities only after long biodegradation periods. Despite the fact that the hydrolytic activities of the two BR species were similar, *Laetiporeus sulfureus* demonstrated a very limited degradative capacity, contrasting with *Wolfiporia cocos*, which induced an effective decay. The amounts of xylanase detected in BR and WR cultures had no correlation with the extent of polyoses removal. However, the fungi providing the highest values of lignin loss were also responsible for the highest values of polyoses removal. This indicates that the lignin removal may have caused wood cell wall permeability, facilitating the xylanases diffusion into and action on the polyoses of the wood cell walls.

Keywords: hydrolytic enzymes; oxidative enzymes; white-rot fungi; brown-rot fungi; wood decay

1. Introduction

The studies on enzyme production by wood rot fungi are usually conducted using chemically defined liquid medium, under conditions able to induce the production of a particular enzyme. However, in natural environments and in solid-state fermentation, these fungi grow on woody substrates under quite different conditions from those of submerged cultures. Several reports have indicated that fungal growth in solid-substrate fermentation is quite different from growth in liquid medium, the patterns of enzyme production being consequently different [1–4].

Through electron microscopy, finer details on the morphological aspects of wood decay by white- and brown-rot fungi can be well understood [5]. However, much research work is still necessary to fully understand the degradation process, and particularly the enzymes and other metabolites secreted by the fungi during wood decay. Information on this could help to elucidate the biochemical mechanisms of wood decay by fungi and consequently facilitate the fungal strain selection for biopulping and for other industrial applications.

Biochemical mechanisms proposed for lignin degradation have been based on the studies of the extracellular ligninolytic systems of basidiomycetes (mainly white-rot fungi), while the polysaccharide degradation by wood-rot fungi has been assumed analogous to the mechanisms used by fungi such as *Trichoderma reesei* and other ascomycetous fungi that efficiently degrade isolated wood polysaccharides [6,7].

This study describes the enzymatic activities produced by two brown- and four white-rot fungi grown on *E. grandis* wood chips under solid-state fermentation. Some relevant hydrolytic (cellulases, β-glucosidases and xylanases) and oxidative (laccases and peroxidases) enzymes present in the fungal extracts from the cultures were studied. In order to investigate the relationship between the enzyme equipment of each fungus and its corresponding pattern of wood decay, changes in the polysaccharides and lignin contents in the decayed wood were also determined.

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2. Materials and methods

2.1. Microorganisms and culture conditions

The brown-rot (BR) fungi *Laetiporeus sulfureus* (ATCC 52600) and *Wolfiporia cocos* (ATCC 62788) and the white-rot (WR) fungi *Poria medula-panis* (ATCC 42463), *Pycnoporus coccineus* (ATCC 32258), *Phlebia tremellosa* (ATCC 48745) and *Trametes versicolor* from the culture collection of the Department of Biotechnology, Lorena, SP (Brazil), were used in this study. The strains were cultivated on 2% malt-extract agar (MEA) plates at 28°C during 5–6 days. To study the enzyme production, Erlenmeyer flasks (2 L) containing 200 ml of 2% MEA medium were prepared. The flasks were inoculated with 4 pieces (approx. 10 mm^2) of agar-mycelium taken from MEA plates and incubated at 28°C during 7 days (time necessary for the mycelium to cover the agar surface).

2.2. Wood decay conditions

Chips from 8-year-old *Eucalyptus grandis* trees (approx. 2.5 × 1.5 × 0.2 cm) were kindly furnished by a local fiberboard mill. The wood chips (50 g), prepared as reported previously [8], were aseptically added to the fungal cultures. The fungal wood decay proceeded at 28°C during 150 days (5 months). The control (uninoculated wood) was prepared by adding the chips to the MEA medium in the same conditions mentioned above, but without inoculum. The cultures were all grown in triplicate.

2.3. Extraction of enzymes from solid-state cultures and weight loss determinations

After 15, 30, 60, 90, 120 and 150 days of incubation, the fungal cultures were sequentially extracted twice using 100 ml of a solution containing 50 mM sodium acetate buffer (pH 5.4) and 0.01% Tween 80. In the first time, the whole culture (MEA medium, colonized wood chips and mycelial mass) was extracted. The wood chips were then separated from the MEA medium and the mycelial mass, and cut in small pieces with a knife (in the direction of the fibers). In the second time, only the pieces of wood chips were extracted. Each extraction lasted 3 h and was carried out in a rotary shaker (120 rpm) at 20 ± 2°C. The content of each flask was filtered through fine filter paper. Crude extracts were maintained at 4°C and the lignocellulolytic enzymes were determined within 2 days after extraction. Extracts obtained from uninoculated wood chips showed no activity of any of the lignocellulolytic enzymes. Extracted wood chips were finally washed with water and dried to constant weight at 105°C. Weight losses were determined on the basis of the initial and final dry weights.

2.4. Enzyme assays

Total cellulase (filter paper activity, FPA) and xylanase were assayed with filter paper Whatman N° 1 and birch xylan, respectively [9,10]. The reducing sugars were determined by using dinitrosalicylic acid (DNS) [11]. Glucose and xylose standard curves were used to calculate the cellulase and xylanase activities, respectively. β-glucosidase was determined by measuring p-nitrophenol released from p-nitrophenyl-β-D-glucopyranoside [12]. Total phenoloxidase and laccase were assayed by oxidation of syringaldazine at pH 5, with and without H_2O_2, respectively [13]. Peroxidase was calculated as the total phenoloxidase minus laccase activity. One unit of activity was defined as the amount of enzyme releasing 1 μmol of product per min (IU). Enzyme activity was expressed as total units recovered from each culture flask after two extractions (units per culture). Enzyme activities were measured in each extract from triplicate cultures. The average and standard deviations of enzymatic activities are shown in Table 1.

2.5. Wood component analyses

Decayed and undecayed wood samples were milled in a knife mill to pass through a 0.5 mm screen. Klason insoluble and soluble lignins were determined in 95% ethanol-extracted milled wood samples by acid hydrolysis as described elsewhere [14]. After acid hydrolysis, soluble sugars were quantified by HPLC using an HPX-87H column eluted with 5 mM sulfuric acid at 0.6 $ml.min^{-1}$. Total polyoses and glucan values derived from calculations considering the acid-released monomers, glucose and xylose [14].

3. Results

3.1. Macroscopic aspects of decayed wood chips

E. grandis wood chips were treated with WR and BR fungi under solid-state conditions. Distinct responses were observed for each fungus in terms of rate of colonization, mycelial mass production and macroscopic aspects of decayed wood. In general, the BR fungi showed a quick rate of wood colonization with a discrete mycelial mass production at an early stage of decay. After 60 days, *W. cocos* produced an abundant mycelial mass on the wood chips, while *L. sulfureus* grew only moderately during the entire period of decay. The wood chips decayed by both BR fungi displayed a great quantity of hyphae on their internal surfaces, which were exposed when the wood was cut for enzyme extraction. After 90 days of biodegradation, all the wood chips showed a reddish-brown color. Those decayed by *W. cocos* were very soft, and when they dried, they became brittle and broke easily into cube-like pieces (transversal fiber sections). On the other hand, the chips rotted by *L.sulfureus* were harder, even at the end of the decay period.

Table 1
Enzymes recovered from solid-state cultures of several white- and brown-rot fungi growing on MEA medium and *Eucalyptus grandis* wood chips*

Fungi and enzymatic activity (IU/culture)	Biodegradation time (days)					
	15	30	60	90	120	150
Wolfiporia cocos						
Total cellulase	8.3 ± 0.4	3.4 ± 0.2	1.4 ± 0.1	4.5 ± 0.3	5.1 ± 0.3	6.3 ± 0.5
β-glucosidase	8.3 ± 0.5	27 ± 2	19.2 ± 0.9	25 ± 1	31 ± 1	42 ± 2
Xylanase	1246 ± 50	101 ± 11	109 ± 10	104 ± 9	928 ± 40	1270 ± 46
Laetiporeus sulfureus						
Total cellulase	15.4 ± 0.9	2.4 ± 0.3	0.80 ± 0.02	2.9 ± 0.1	4.5 ± 0.2	7.2 ± 0.5
β-glucosidase	7.6 ± 0.7	22 ± 1	13.0 ± 0.8	17.0 ± 0.8	37 ± 2	23 ± 2
Xylanase	958 ± 35	127 ± 11	73 ± 7	90 ± 9	85 ± 8	1100 ± 49
Poria medula-panis						
Total cellulase	3.4 ± 0.6	1.40 ± 0.09	0.40 ± 0.01	2.6 ± 0.6	2.0 ± 0.2	2.5 ± 0.1
β-glucosidase	2.7 ± 0.2	10.5 ± 0.5	9.4 ± 0.8	8.6 ± 0.6	8.7 ± 0.5	5.5 ± 0.5
Xylanase	335 ± 20	37 ± 3	31 ± 3	32 ± 3	48 ± 4	53 ± 5
Peroxidase	5.2 ± 0.4	3.7 ± 0.3	2.5 ± 0.2	4.3 ± 0.3	5.3 ± 0.4	1.5 ± 0.4
Laccase	0.6 ± 0.3	6.8 ± 0.5	2.5 ± 0.1	0.80 ± 0.02	0.70 ± 0.05	0.50 ± 0.01
Pycnoporus coccineus						
Total cellulase	3.7 ± 0.3	1.8 ± 0.1	1.20 ± 0.09	2.9 ± 0.1	2.9 ± 0.2	3.6 ± 0.2
β-glucosidase	8.0 ± 0.7	22 ± 2	19 ± 2	14.5 ± 0.9	14 ± 1	9.1 ± 0.7
Xylanase	486 ± 33	66 ± 6	52 ± 5	71 ± 5	58 ± 4	99 ± 9
Peroxidase	0.70 ± 0.05	5.1 ± 0.3	0.0	3.1 ± 0.4	2.5 ± 0.2	5.8 ± 0.3
Laccase	7.0 ± 0.6	18 ± 1	21 ± 1	9.9 ± 0.7	8.8 ± 0.5	12.0 ± 0.8
Phlebia tremellosa						
Total cellulase	0.0	2.00 ± 0.08	0.80 ± 0.01	1.70 ± 0.07	1.60 ± 0.09	0.60 ± 0.01
β-glucosidase	3.8 ± 0.2	15.6 ± 0.9	6.0 ± 0.5	6.5 ± 0.5	5.4 ± 0.3	8.9 ± 0.5
Xylanase	542 ± 33	94 ± 8	52 ± 5	123 ± 10	111 ± 11	76 ± 7
Peroxidase	2.4 ± 0.1	3.5 ± 0.3	1.5 ± 0.1	2.7 ± 0.1	0.30 ± 0.01	0.80 ± 0.03
Laccase	2.5 ± 0.2	5.2 ± 0.4	4.2 ± 0.3	5.1 ± 0.3	0.90 ± 0.02	9.3 ± 0.8
Trametes versicolor						
Total cellulase	4.0 ± 0.3	1.5 ± 0.1	0.80 ± 0.07	1.00 ± 0.09	1.6 ± 0.1	2.6 ± 0.2
β-glucosidase	3.8 ± 0.4	20 ± 1	42 ± 1	8.9 ± 0.7	12 ± 0.6	15.4 ± 0.9
Xylanase	335 ± 26	85 ± 8	107 ± 10	82 ± 8	116 ± 11	95 ± 6
Peroxidase	0.70 ± 0.05	6.2 ± 0.5	0.0	0.80 ± 0.06	2.0 ± 0.1	1.30 ± 0.07
Laccase	4.7 ± 0.4	17 ± 1	39 ± 1	3.0 ± 0.2	7.6 ± 0.5	4.4 ± 0.4

* Each culture contained 200 mL of 2% malt extract agar medium plus 50 g of *Eucalyptus grandis* wood chips. Enzymatic activities are the total activity recovered from each culture after two extractions (see M&M).

E. grandis wood chips were quickly colonized by the WR fungi (except for *P. tremellosa*) with the formation of abundant mycelial mass. At advanced stages of decay, the coloration of the chips was whitish-yellow or whitish-tan, depending on the fungus. The decayed chips were soft, brittle and spongy or easily divided into longitudinal fragments, principally the chips decayed by *T. versicolor*. Several black spots and flecks were observed in the chips decayed by *P. medula-panis, P. coccineus* and *T. versicolor*. These spots, located on the inner and outer surfaces of the chips, have been associated with manganese dioxide deposits produced by some fungi [15].

3.2. Enzyme extraction from solid-state cultures

The recovery of enzymes from solid substrates like wood is difficult, owing to the pore-filled structure of the substrate and the nature of the proteins that can be aggregated into the cell lumens [1,16,17]. It can be assumed that the enzymes detected in the first extract were enzymes that diffused into the MEA medium during the decay period, or enzymes associated with fungal mycelium and/or with exposed hydrated surfaces of wood chips. Moreover, the enzymes detected in the second extract could be enzymes associated with the inner surfaces of decayed chips, which were exposed when the chips were cut. The second extraction yielded total enzymatic activities in the range of 5–15% of the first extraction. The sum of enzymatic activities recovered in both extractions was considered proportional to the total enzymes produced during decay. The total enzymatic activities detected by means of these extraction procedures are shown in Table 1. As the decay experiments included the use of malt extract as a co-substrate for fungal growth, several enzymes detected during the initial decay periods, which were possibly produced at the expense of the malt-extract-agar (MEA) medium, contributed to the total units of activities of each culture.

Both groups of fungi (BR and WR) produced hydrolytic activities during the entire biodegradation process, BR presenting the highest values. *W. cocos* and *L. sulfureus*

showed the highest cellulase and xylanase activities not only in the first 15 days but also at advanced stages of decay (Table 1). In general, *W. cocos* showed higher xylanase and β-glucosidase levels than *L. sulfureus*, but the cellulase activities produced by the latter reached the highest levels.

The levels of cellulase activity produced by the WR fungi were similar over all the decay period, while the levels of xylanase activity varied, reaching their maximum in *P. tremellosa* cultures on the 15th day of biodegradation.

The levels of β-glucosidase activity detected in the WR and BR extracts were similar. However, the biodegradation time necessary for the fungi to produce maximal levels of this enzyme was not the same for the two groups. While the BR fungi needed 120–150 days of decay to reach their peaks, WR needed only 30–60 days, depending on the species.

Lignin-peroxidase activity could not be assayed, because UV-absorbing compounds derived from wood and present in the fungal extracts interfered with the veratryl alcohol method. It was also difficult to distinguish manganese-dependent peroxidase from other peroxidases, since the extracts might contain trace levels of Mn^{2+} derived from *E. grandis* wood and MEA medium. Therefore, two main groups of phenoloxidases were determined in each extract: laccases and peroxidases, both using syringaldazine as the model substrate. These ligninolytic activities were detected in all the WR fungal extracts, but not in the BR extracts.

Laccase activity reached maximum levels at early decay stages (30 or 60 days depending on the fungus), decreasing afterwards. In the case of *P. tremellosa*, however, the laccase activity reached its peak at the end of decay (Table 1).

The levels of peroxidase activity in the extracts were always lower than the levels of laccase, except for *P. medula-panis*. Interestingly, *P. coccineus* and *T. versicolor* showed similar peroxidase profiles with no activity on the 60th day, when the maximal laccase peak was attained (Table 1). These fungi also showed the highest levels of total phenoloxidase (laccase plus peroxidase) during the whole period of decay.

3.3. Weight and component losses of decayed wood

The curves of weight and component losses of decayed *E. grandis* wood chips are shown in Fig. 1. The two BR fungi differ significantly in their capacity for wood degradation. *L. sulfureus* proved to be a much slower degrader than *W. cocos*, bringing about very low losses of glucan and polyoses and failing to degrade lignin. Conversely, the weight loss caused by *W. cocos* increased gradually after 15 days, reaching 37% on the 150th day. This fungus produced typical brown-rot decay, glucan and polyoses being the main components degraded. However, lignin removal was also significant at advanced decay stages (120 and 150 days), even when *W. cocos* extracts did not show phenoloxidase activity. Thus, the weight and component losses caused by these two BR fungi could be correlated with macroscopic aspects of decayed wood, but the similar high levels of hydrolytic activities produced by these fungi can not explain the great differences in their capacities to degrade polysaccharides.

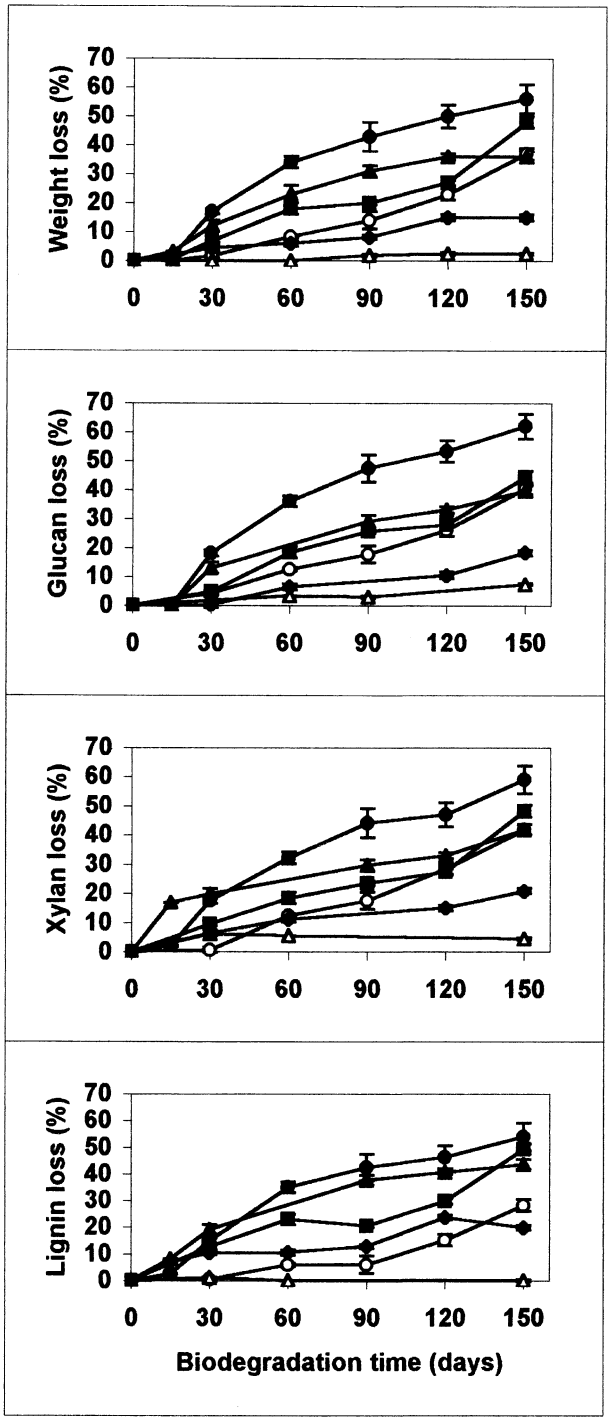

Fig. 1. Weight and component losses of *Eucalyptus grandis* wood chips decayed by white- and brown-rot fungi under solid-state fermentation. (-o-) *Poria cocos*, (-Δ-) *Laetiporus sulfureus*, (-▲-) *Phebia tremellosa*, (-♦-) *Poria medula-panis*, (-■-) *Pycnoporus coccineus*, (-●-) *Trametes versicolor*. Error bars represent deviation from the average value of the sample and where not shown are smaller than the symbol.

WR fungi also demonstrated differences in their capacities for wood degradation, but not to the same degree as BR fungi. The highest values of weight loss (56% and 48%) were provided by *T. versicolor* and *P. coccineus*, respectively and the lowest (15%) by *P. medula-panis* (Fig. 1). In general, *T. versicolor* showed the highest ability to degrade all the wood components, causing elevated losses of glucan (62%), polyoses (59%) and lignin (54%) on the 150th day of biodegradation. *P. medula-panis*, *P. coccineus* and *P. tremellosa* removed more lignin than glucan within periods no longer than 30 days. After 30 days of culturing, these fungi started to remove similar amounts of lignin and glucan, showing a typical nonselective pattern of degradation.

4. Discussion

BR and WR fungi produced several enzymes when *E. grandis* wood chips were treated under solid-state fermentation. The profiles of extracellular enzymatic activities produced during wood decay varied among the fungi studied, but some general features were noted. All the fungi produced hydrolytic activities, but BR fungi produced higher levels of cellulase and xylanase than WR fungi. On the other hand, phenoloxidases were only found in WR fungal extracts. Of the lignocellulolytic enzymes assayed, xylanase was detected in the highest quantity, in terms of total units per culture.

The high levels of enzymatic activities detected after 15 days of decay did not correlate with the extent of initial wood weight or component losses. This indicates that the enzymes (produced probably at the expense of the MEA medium) did not degrade the wood components effectively, probably owing to their low permeation into the wood cell walls [18]. On the other hand, the enzymatic activities detected after longer biodegradation periods provided some evidences of the different decay patterns observed from each fungus. *T. versicolor* and *P. coccineus*, which produced the highest final lignin loss values, were also the best producers of oxidative enzymes, especially laccase. However, in the case of *P. tremellosa*, a significantly lower total phenoloxidase activity was followed by lignin loss values close to those observed in *T. versicolor* and *P. coccineus* cultures. A significant lignin removal was also observed at advanced stages of wood decay by *W. cocos* even when there was no phenoloxidase activity. These results indicate that different lignin biodegradation mechanisms might have been involved in each case. Regarding *P. tremellosa* cultures, low total phenoloxidase activities followed by high lignin removal from the wood chips could be related to the involvement of more efficient peroxidases, such as LiP, in the lignin biodegradation. Some non-enzymatic mechanisms of lignin biodegradation [19] might also explain the large extent of lignin degradation even with low or absent phenoloxidase activity, as in the case of *P. tremellosa* and *W. cocos*, respectively.

The high hydrolytic activities detected at advanced stages of decay in *W. cocos* cultures correlate well with the polysaccharide degradation and with the weight losses observed. However, *L. sulfureus*, which was a much slower degrader than *W. cocos*, presented similar hydrolytic enzyme levels. These data suggest that *L. sulfureus* has a limited capacity to permeate wood cell walls for enzyme diffusion and action. A similar conclusion arises from data on polyoses losses and total xylanase activity detected in the cultures of both WR and BR fungi. The highest polyoses losses were observed in the *T. versicolor*, *P. coccineus* and *P. tremellosa* cultures. However, these fungi were not the best producers of xylanase. The xylanase activities detected in the cultures of these fungi at advanced stages of decay (after 90 days of biodegradation) were almost 10 times lower than the activities detected in the cultures of BR fungi. Interestingly, *T. versicolor*, *P. coccineus* and *P. tremellosa* were also the best lignin degraders, which indicates that lignin removal could provide wood cell wall permeability, facilitating the diffusion and the action of the hydrolytic enzymes. With respect to the WR fungi, the cellulase production also did not correlate with glucan removal. In fact, the levels of total cellulase produced by *P. medula-panis* were similar to those produced by *T. versicolor* and *P. coccineus*, although the glucan degradation was low, following the same pattern of lignin loss.

In conclusion, the results indicate that the production of high enzymatic activities did not necessarily result in a large extent of a specific wood component removal. On the other hand, lignin removal (either by enzymatic or by hypothetical non-enzymatic mechanism) confirmed to be a key processes for an overall wood decay. This indicates that the lignin removal may have caused wood cell wall permeability, facilitating the enzymes diffusion into and action on the of the wood cell walls, the extent of polysaccharide degradation being closely dependent of the extent of lignin removal.

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