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ORIGINAL

Effect of heat treatment on extracellular enzymatic activities involved in beech wood degradation by *Trametes versicolor*

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Abstract Effect of heat treatment on extracellular enzymes involved in wood degradation by *Trametes versicolor* was investigated. Heat-treated and untreated beech blocks were exposed to *T. versicolor* on malt-agar medium and extracellular enzymatic activities investigated. A strong ABTS oxidizing activity has been detected during the first stage of colonization in both cases, while cellulase activities are mainly detected in the case of untreated beech wood. Further investigations carried out on holocellulose, isolated using sodium chlorite delignification procedure and subjected to heat treatment or not, indicate that commercially available cellulases and xylanases are able to hydrolyse untreated holocellulose, while heat-treated holocellulose was not affected. All these data suggest that chemical modifications of wood components during heat treatment disturb enzymatic system involved in wood degradation.

Introduction

Adoption of Biocidal Product Directive in 1998 has lead to important changes in the field of wood preservation in Europe. Different heat treatment processes have been developed during the last decade allowing industrialisation and commercialization of heat-treated timbers resulting from the treatment of low natural durability wood

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species (Patzelt et al. 2002; Militz 2002). Heat treatment carried out under inert atmosphere of nitrogen in a temperature range between 200 and 260°C converts wood into a new product called torrified or rectified wood (Weiland and Guyonnet 2003). Heat-treated wood possesses new properties like improved dimensional stability and decay resistance, while its strength decreases more or less according to the treatment conditions (Tjeerdsma et al. 1998; Santos 2000; Alén et al. 2002; Kamdem et al. 2002; Pétrissans et al. 2003; Gosselink et al. 2004; Hakkou et al. 2005, 2006; Yildiz et al. 2006). Due to the improvement of durability towards wood rotting fungi, heat-treated wood is considered as a valuable "non-biocidal" alternative to classical wood preservatives for applications in hazard classes 2 and 3. However, in spite of numerous studies on the reasons of this improvement of durability of heat-treated wood (Weiland and Guyonnet 2003; Alén et al. 2002; Kamdem et al. 2002; Gosselink et al. 2004; Hakkou et al. 2006; Boonstra et al. 2006), no data has been reported on the effect of chemical modifications occurred during treatment on fungal enzymatic activities involved in wood degradation. The aim of this paper is to investigate the effect of heat treatment on the production of different enzymatic activities, like cellobiohydrolases, β-glucosidases and oxidases involved in carbohydrates and lignin degradation during wood colonization and degradation by the white-rot fungus *Trametes versicolor*.

Materials and methods

Heat treatment

Wood heat treatment was performed under nitrogen on previously dried beech blocks (*Fagus sylvatica*) ($15 \times 30 \times 35$ mm^3, R × T × L) in a glass reactor placed in an oven at 240°C for 8 h. The oven temperature was increased by 20°C min^{-1} from ambient to final temperature. Mass loss of the samples after heat treatment was equal to 22% ± 0.5.

Decay fungus

The white rot fungus, *Trametes versicolor* (L) Quelet (Strain CTB 863A) was used in this study. Stock cultures of fungi were maintained on malt-agar slants stored at 4°C.

Solid state cultures on wood

Beech wood blocks ($15 \times 5 \times 35$ mm^3, R × T × L) were dried at 103°C and weighed at ±0.01 g (m_0). Sterile culture medium (20 ml), prepared from malt (15 g) and agar (15 g) in distilled water (1 l), was placed in 9 cm Petri dishes, inoculated with fungus and incubated for 2 weeks at 22°C and 70% HR to allow colonization of the medium by the mycelium. Five blocks (heat-treated and untreated) were placed in each Petri plates under sterile conditions. Incubation was carried out at 22°C under controlled humidity conditions of 70 RH in a climatic chamber WTB BINDER TYP KBF 115 (Tuttlingen, Germany).

After different incubation times, colonized wood blocks were carefully collected, mycelium removed, dried at 103°C and weighed (m_1). Weight loss (WL) was expressed as a percentage of the initial oven dried weight of the sample according to the following formula, where m_0 corresponds to the initial mass of the wood block:

$$\mathrm{WL}(\%) = ((m_0 - m_1)/m_0) \times 100$$

Enzymatic activities using fluorogenic assays

Colonized wood blocks were carefully removed from agar plates, mycelium surrounding wood blocks being excised. Each wood block covered on its surface with mycelium was crushed and placed into a test tube containing 1 ml of 50 mM phosphate buffer pH 6. Then, mycelium was separated from the wood in order to facilitate enzyme extraction and the whole samples were incubated for 1 hour at room temperature. The samples were then centrifuged (10,000×*g* for 15 min) and the supernatant collected to test the different activities. Each extraction was repeated nine times with nine different blocks after each incubation period and the values were averaged.

Three enzymes substrates based on methylumbelliferone (MU) were used for enzymatic detections: MU-*β*-D-glucopyranoside for *β*-glucosidase (EC 3.2.1.3), MU-*β*-D-cellobioside for cellobiohydrolase (EC 3.2.1.91) and MU-N-acetyl-*β*-D-glucosaminide (MU-NAG) for chitinase. All chemicals were purchased from Sigma Aldrich SARL (Saint Quentin Fallavier, France). The experimental conditions for fluorescence assays, incubation buffers and substrates concentrations were the same as described previously (Lekounougou et al. 2007). Briefly, 50 µl of samples were mixed with 50 µl substrate solution, and 50 µl incubation buffer according to Courty et al. (2005). Incubation occurred in black 96-well microtitration plates for 1 h, fluorescence emission being recorded every 5 min on a Cary Eclipse Fluorescence Spectrophotometer (Varian, Mulgrave, Australia) with an excitation wavelength of 360 nm and an emission wavelength of 450 nm.

ABTS oxidizing activities

ABTS oxidizing activities were monitored as described previously (Lekounougou et al. 2007). Colonized wood blocks were removed carefully from agar plates and mycelium surrounding wood blocks were excised. Each wood block was crushed and then placed into a test tube containing 900 µl of 50 mM phosphate buffer pH 6 and the mycelium was separated from the wood prior to addition of 50 µl of 100 mM 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonat. In order to stay in substrates-saturated conditions, incubation times were varied (typically, from 10 min to 2 h) in order to obtain a 420 nm OD around 0.5. The samples were then centrifuged (10,000×*g* for 5 min) before determination of the supernatant absorbance at 420 nm ($\varepsilon_{420} = 36\ \mathrm{mM}^{-1}\ \mathrm{cm}^{-1}$). Each extraction was repeated nine times after each incubation period and the values were averaged. Oxidizing activity is expressed in International Unit (UI) corresponding to mmoles of substrate transformed per minute. The specific activity is expressed in UI per mg of dry wood.

Preparation of holocellulose and of heat-treated holocellulose

Beech heartwood was grounded to fine sawdust with a Siebtechnik vibratory disc mill and passed through different sieves to obtain a granulometry between 0.2 and 0.5 mm. The sawdust, thus obtained, was washed in a Soxhlet extractor with a toluene/ethanol (2:1, v/v) mixture (6 h), with ethanol (6 h) and dried at 103°C for 48 h before delignification. 500 mg of sawdust were placed in a 100 ml flask containing 30 ml of distilled water and heated at 75°C. Acetic acid (0.1 ml) and 15% aqueous sodium chlorite (2 ml) were then added each hour for 7 h. The mixture was filtered on a Büchner funnel and the residue washed with water, Soxhlet extracted for 2 h with ethanol and dried at 103°C to a constant mass. Heat treatment of hollocellulose was performed under nitrogen at 240°C for 5h30 as described previously (Nguila Inari et al. 2007).

Cellulase and xylanase assays

Cellulase from *Trichoderma reesei* (ATCC 26921) and xylanase from *Thermomyces lanuginosus* (EC 253-439-7) were purchased from Sigma Aldrich SARL (Saint Quentin Fallavier, France). Fifty micro grams of cellulase or 125 mg of xylanase were placed into test tubes containing 10 ml of 50 mM acetate buffer (pH 5). Approximately 230 mg of heat-treated or untreated holocellulose (m_i) were added in each tube and the mixture was incubated for 24 or 72 h at 37°C for cellulase and xylanase, respectively. The remaining holocellulose was separated from the hydrolysate by centrifugation (10,000×g for 30 min). The residue was filtered, washed with distilled water, dried at 103°C and weighed (m_f). Weight loss due to enzyme activity was calculated according to the following formula:

$$\mathrm{WL}(\%) = ((m_i - m_f)/m_i) \times 100$$

Results and discussion

Methodology used to perform solid state cultures on wood has been adapted from EN-113 (1986) standard. Due to the aim of the study, which was to determine enzymatic activities, the size of wood blocks was reduced to improve fungal degradation and to be more consistent with enzymatic assays generally performed on small size samples. The mean percentage weight losses of heat-treated or untreated samples exposed to *T. versicolor* for different periods are given in Fig. 1.

After 90 days of incubation, wood weight losses of around 45% were measured for untreated samples confirming the virulence of *T. versicolor* under the tested conditions. In all cases, control samples were deeply colonized by the mycelium, while heat-treated samples were only slightly or not colonized. Weight losses recorded for heat-treated samples were low and consistent with values previously reported under similar conditions (Hakkou et al. 2005). Decaying process of untreated beech wood appeared to proceed in two stages leading to different wood weight loss rates. During the first 10 incubation days, measured weight losses were too small to determine significant wood degradation. Between 10 to around 40

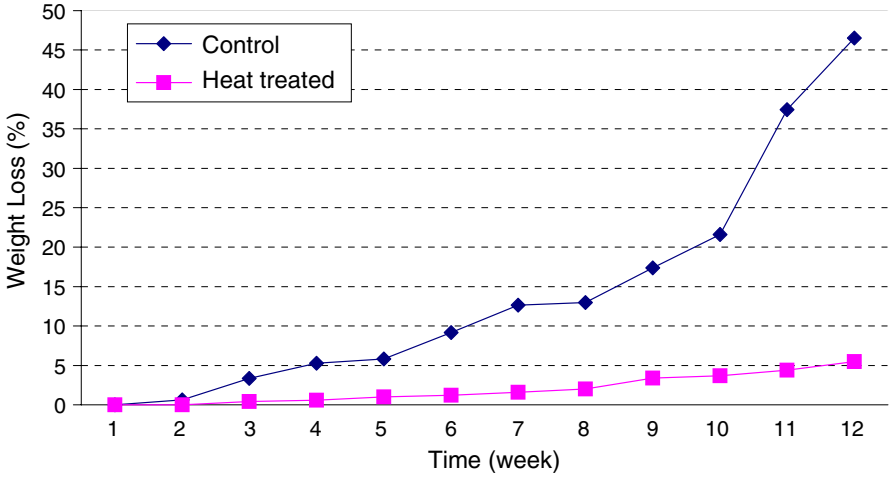

Fig. 1 Weight losses of wood blocks exposed to *T. vesicolor*

incubation days, the apparent decay rate appeared to be more or less constant and corresponded to a weight loss value of approximately 0.35% per day. After 45 incubation days, this degradation rate increased to a value of 0.80% per day.

The ABTS oxidases production pattern during wood degradation by *T. versicolor* is shown in Fig. 2.

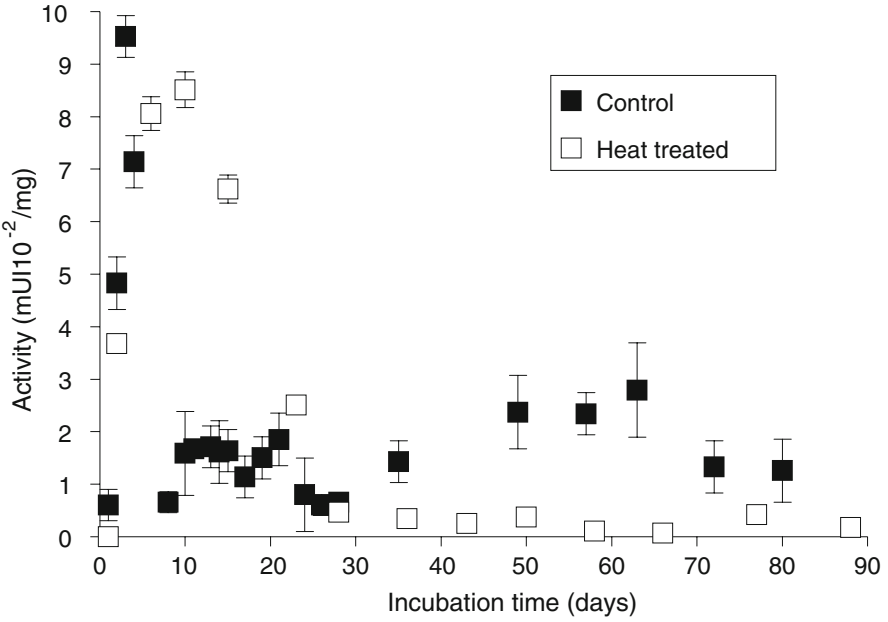

Fig. 2 ABTS oxidizing activities during wood colonization by *T. versicolor*. Each point is the mean ± standard deviation of nine extractions performed on nine different blocks

Behaviour of ABTS oxidizing activities was different according to the wood substrate used. In the case of untreated samples, three stages could be observed during the whole process. In the first 10 incubation days, a strong activity was observed, reaching around 0.07 mUI mg^{-1} after 4 days. This activity has been previously associated with laccases production, which are involved in wood extractives degradation (Lekounougou et al. 2007). In the second stage, comprised between 10 and about 30 incubation days, a second increase of the ABTS oxidizing activity has been detected. Nevertheless, the maximal value obtained during this stage remains largely smaller than the one detected during the first part (around 0.02 mUI/mg). The third increase of activity started after around 45 days and seems to be correlated with the increase of the wood degradation rate (see Fig. 1). The maximal value of activity detected during this phase was around 0.03 mUI/mg after 60 days followed by a slight decrease. In the case of heat-treated samples, oxidizing activities were detected only during the 30 first days of incubation and are practically inexistent after this period. The maximal activity detected for heat-treated samples (0.08 mUI/mg) was similar to that detected in the first stage of control colonization. Carbohydrates hydrolytic activities have also been investigated using specific substrates for cellobiohydrolases, β-glucosidases responsible of cellulose degradation. Results are presented in Figs. 3 and 4. Similar patterns were observed for the two tested activities in the case of untreated beech blocks. A first stage of degradation, characterized by a relatively low and constant activity, was detected between 0 and 40 incubation days followed by a second stage corresponding to a strong increase of activity. However, even if *T. versicolor* is known to degrade simultaneously lignin and polysaccharides (Machuca and Ferraz 2001), detection of enzymatic activities involved in degradation of these two components seems quite different. Contrary to peroxidases and laccases, carbohydrates degrading enzymes could not act *via* low molecular weight mediators, a previous lignin removal being necessary to permit these enzymes to diffuse into the cell walls (Cullen and Kersten 2004; Machuca and Ferraz 2001). This could explain the low level of cellulases and hemicellulases detected during the first stage of colonization. Another important point is that the fungal production of cellulases and hemicellulases has been shown to be tightly regulated depending on both presence of inducing agents and absence of easily available carbon source (Aro et al. 2005). Therefore, the increase of wood degradation rate observed after 40 incubation days could be also due to a depletion of easily assimilable malt-agar nutriments. This hypothesis is supported by the induction at the same time of chitinases activities, which could be involved in nutrients mobilization during the second part of the experiment (Fig. 5). In fungi, chitinases could have different functions depending on their localization. The extracellular chitinases are mainly involved in extracellular chitin degradation, a way to obtain additional carbon and nitrogen sources. In contrast, cell wall associated chitinases are mainly involved in processes regulating cell wall dynamics, their action being required during fungal growth and also during autolysis (Bowman and Free 2006). The increase of detected chitinases in our experimental systems occurs simultaneously with the induction of cellobiohydrolases, proteases and phosphatases (Lekounougou et al. 2008), suggesting an exhaustion of malt-agar nutriments. Furthermore, during the induction of these

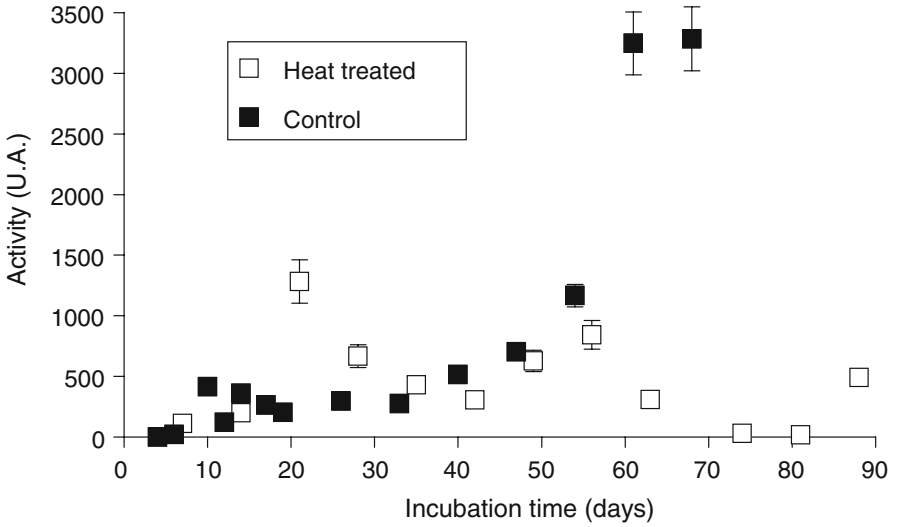

Fig. 3 Glucosidase activities during wood colonization by *T. versicolor*. Each point is the mean ± standard deviation of nine extractions performed on nine different blocks

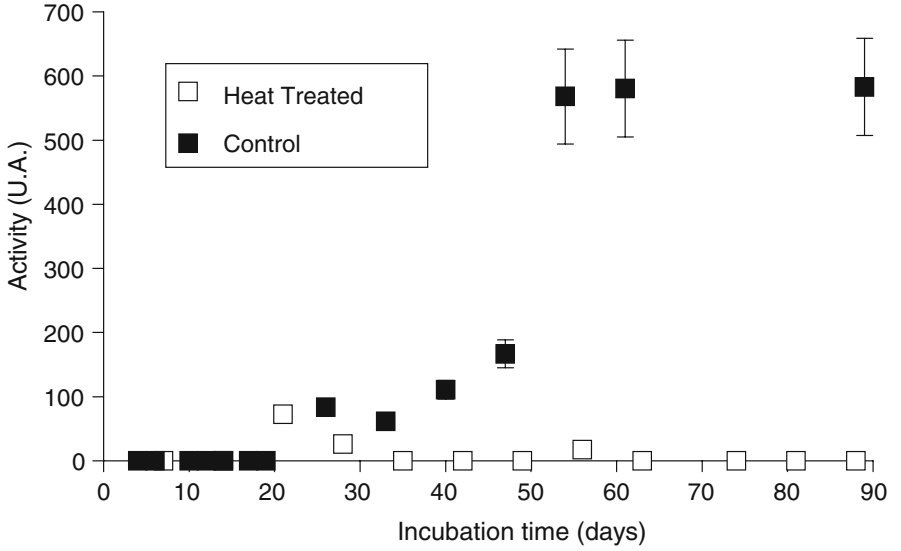

Fig. 4 Cellobiohydrolase activities during wood colonization by *T. versicolor*. Each point is the mean ± standard deviation of nine extractions performed on nine different blocks

enzymes, the wood degradation still occurred. Taking together these data suggests that the high increase of detected activities results from the expression of extracellular enzymes involved in nutrients mobilization. Nevertheless, the involvement of cell wall associated enzymes (chitinases and β-glucosidases) in

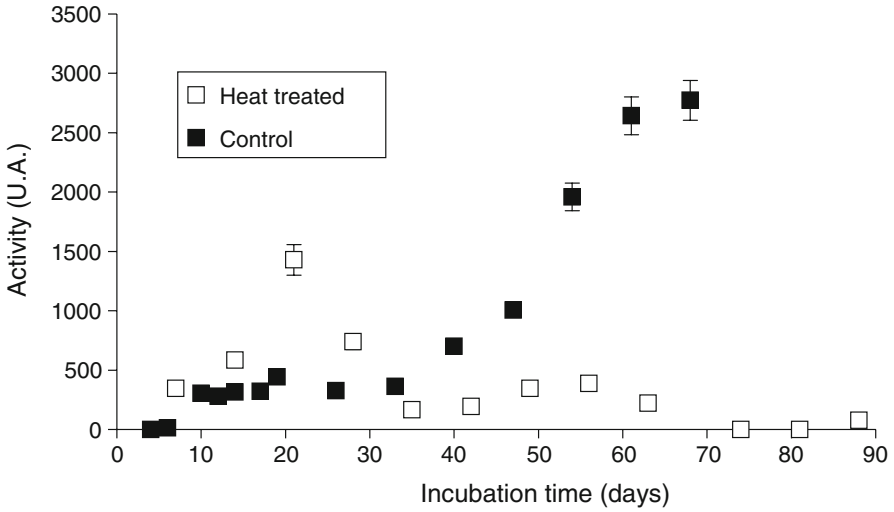

Fig. 5 Chitinase activities during wood colonization by *T. versicolor*. Each point is the mean ± standard deviation of nine extractions performed on nine different blocks

the detected activities cannot be excluded totally, resulting from autolysis phenomenon, a process occurring also during nutriments depletion (Nijikkena et al. 2007).

The behaviour of heat-treated beech is completely different leading to the detection of very low levels of hydrolytic activities. Glucosidase activities detected in the presence of heat-treated beech wood are similar to that detected in the first stage of degradation of untreated wood and remains constant throughout the experiment, while practically no cellobiohydrolase activities were detected. Chitinase activities detected during degradation of heat-treated wood are important in the first part of the experiment and decrease with time during the second part of the latter ones. All these results clearly demonstrated that the production of enzymatic activities during wood decaying process by *T. versicolor* is strongly connected to the nature of the wood blocks used. Heat treatment results in a noticeable decrease of carbohydrates hydrolytic activities like cellobiohydrolases and β-glucosidases, while ABTS oxidizing activities involved in lignin degradation seem less affected. These observations could be directly connected to the evolution of wood chemical composition during heat treatment. Indeed, it is well established that heat treatment resulted in an important degradation of amorphous polysaccharides constitutive of the cell walls, while lignin and crystalline cellulose remain unaffected (Tjeerdsma et al. 1998; Sivonen et al. 2002; Wikberg and Maunu 2004; Windeisen et al. 2007). These modifications have been reported to originate from the changes of some macroscopic properties of the material. Dimensional stability and decay resistance increased, while the strength of wood decreased (Santos 2000; Alén et al. 2002; Kamdem et al. 2002; Pétrissans et al. 2003; Gosselink et al. 2004; Hakkou et al. 2005, 2006; Yildiz et al. 2006). Different reasons can be proposed to explain improvement of wood durability to fungi:

- an increase of the hydrophobic character of wood limiting the sorption of water and consequently the development of fungi.
- generation of biocidal extractives during heat treatment which can act as fungicides.
- modification of the wood polymers leading to a non recognition of the latter by enzymes involved in fungal degradation and especially
- degradation of hemicelluloses, which constitute an important nutrient source in the initial stages of cell wall decay.
- reduction in cell wall porosity due to higher dimensional stability of the material after treatment, which makes it more difficult for enzymes to penetrate into the cell wall.

According to the preceding results, it seems that the modification of wood polymers during heat treatment considerably disturbs enzymatic systems involved in wood biodegradation. These modifications are particularly obvious in the case of enzymes involved in polysaccharides degradation, while enzymes involved in lignin degradation seem less affected. However, the absence of enzymatic activities could also be associated to generation of fungicidal extractives during treatment. To confirm the effect of chemical modification on enzymatic activities, the behaviour of holocellulose heat-treated or not has been investigated in vitro using commercially available cellulase and xylanase (Fig. 6). The results indicated that holocellulose was totally solubilized after 24 h incubation in the presence of cellulase, while heat-treated holocellulose was only partially solubilized under the

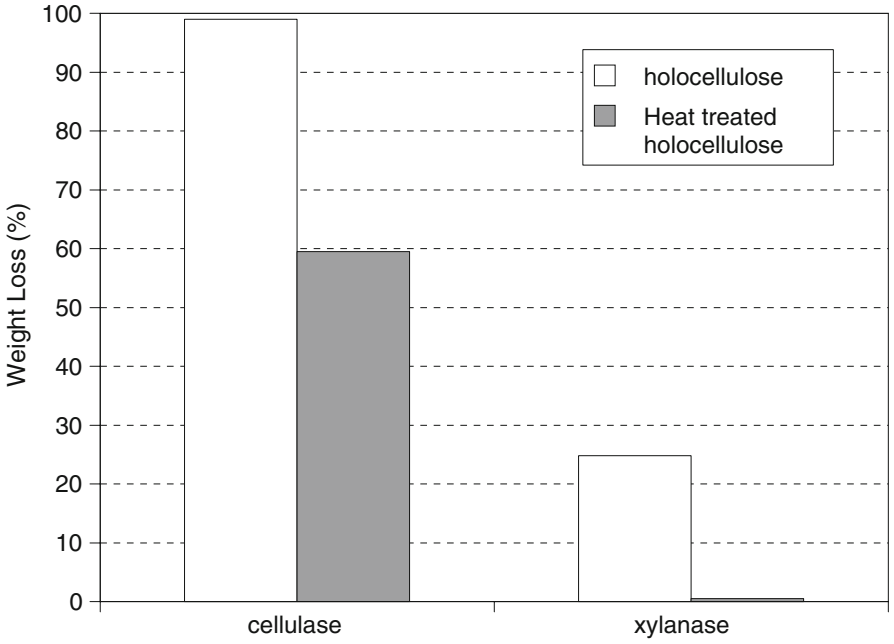

Fig. 6 In vitro degradation of holocellulose by different commercially available enzymes

same conditions. These results confirm that chemical modification of wood polysaccharides disturb enzymatic systems involved in wood degradation. It seems therefore evident that degradation of less ordered polysaccharides has a crucial effect on wood durability. These results are consistent with the explanation concerning the importance of hemicelluloses as nutrient source in the initial stages of cell wall decay or that concerning reduction of cell wall porosity, which can limit diffusion of cellulolytic enzymes into the cell walls. Similar results were obtained with xylanases, which are able to partially degrade holocellulose, while heat-treated holocellulose remains unaffected under the same conditions. Even if thermal behaviour of isolated holocellulose can be different from that of the polysaccharides within the cell wall, these results clearly indicated that thermally modified hollocellulose is less susceptible to enzymes involved in polysaccharides degradation explaining improved durability of heat-treated beech wood.

Conclusion

Production of extracellular enzymatic activities has been studied during wood degradation by *T. versicolor* using either heat-treated or untreated beech blocks. Oxidases production pattern indicate a strong activity in both cases at the beginning of wood colonization. This increase is associated with the production of laccases which are involved in extractives degradation. After this first stage, oxidases production is less important in the case of untreated beech blocks and decrease significantly in the case of heat-treated beech blocks. Carbohydrates hydrolytic activities (cellobiohydrolases and β-glucosidases) have been detected mainly in the case of untreated beech blocks, with a strong increase of activities after about 40 days of incubation. This increase of carbohydrates hydrolysing activities is correlated with the increase of wood weight losses as well as with increase of chitinases activities involved in nutrients mobilization. Additional experiments performed on heat-treated or not holocellulose subjected to commercially available cellulase and xylanase indicated that heat-treated holocellulose is less susceptible to enzymatic hydrolysis than untreated one. All these data suggest that chemical modifications resulting from heat treatment disturb enzymatic systems involved in wood degradation by *T. versicolor* and could progonate from the improvement of durability observed for heat-treated wood. These observations are also in good agreement with the fact that improvement of wood durability can be achieved only if a sufficient level of chemical modification has been reached.

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