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Lignocellulolytic activity of *Coniophora puteana* and *Trametes versicolor* in fermentation of wheat bran and decay of hydrothermally modified hardwoods

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ABSTRACT

In submerged- (SF) and solid-state fermentation (SSF) of wheat bran (WB) substrate, the brown-rot fungus *Coniophora puteana* produced high levels of endoglucanase, xylanase and filter paper activity (FPA) but neither laccase nor manganese-dependent peroxidase (MnP) activity was detected. SF favored the accumulation of endoglucanase (3.2 U ml^{-1}), xylanase (9.4 U ml^{-1}), and FPA (0.14 U ml^{-1}) by *C. puteana*. Under the same cultivation conditions, the white-rot fungus *Trametes versicolor* secreted lower levels of hydrolytic enzymes but expressed high laccase and MnP activities in SF (0.93 U ml^{-1} and 1.75 U ml^{-1}, respectively) and SSF (1.54 U ml^{-1} and 1.63 U ml^{-1}, respectively) of WB. Extracellular enzymatic activities and weight loss (WL) were investigated in untreated and hydrothermally modified (HTM) birch, aspen, and alder wood blocks, exposed to *C. puteana* and *T. versicolor*. Acetone soluble extractives and cellulose content were determined in HTM hardwoods colonized by *C. puteana*. WL and cellulose degradation in untreated wood correlated with the high levels of cellulase activity produced by *C. puteana*. HTM noticeably increased decay resistance, although it was not toxic to the fungal growth and did not disturb the secretion of the enzymes involved in wood degradation.

1. Introduction

Lignocellulose is the major structural component of wood and represents a major source of renewable organic matter. Wood-rotting fungi are the most efficient and extensive lignocellulose degraders due to their capability to produce a variety of hydrolytic and oxidative enzymes acting with various specificity and synergy (Highley and Dashek, 1998; Baldrian and Valaskova, 2008). The major hydrolytic enzymes are endo-1,4-β-glucanase (EC 3.2.1.4; endocellulase), cellobiohydrolase (CBH, EC 3.2.1.91; exocellulase), β-glucosidase (EC 3.2.1.21) and xylanase (EC 3.2.1.8). White-rot fungi employ an array of extracellular hydrolases (Teeri, 1997) that attack cellulose and hemicelluloses and secrete one or more of three extracellular enzymes, which are essential for lignin degradation, namely, lignin peroxidase (EC 1.11.1.14), manganese-dependent peroxidase (EC 1.11.1.13) and laccase (EC 1.10.3.2), and can fully mineralize all cell wall polymers in hardwood decay (Martinez et al., 2005; Lundell et al., 2010). Brown-rot fungi, predominant in softwoods, firstly depolymerize cellulose chains (Kaneko et al., 2005; Suzuki et al., 2006; Howell et al., 2009) and chemically modify lignin (Eriksson et al., 1990; Mai et al., 2004; Yelle et al., 2008; Irbe et al., 2011) by employing non-enzymatic mechanisms. Secondly, enzymatic cleavage of cellulose and hemicelluloses through the use of an incomplete cellulase system occurs. In most cases, brown-rot fungi do not produce the full complex of cellulose-degrading enzymes such as cellobiohydrolases, needed to enzymatically hydrolyze crystalline cellulose (Arantes et al., 2012). However, there are some reports that *Coniophora* brown-rotters (Highley, 1988) and *Gloeophyllum trabeum* (Cohen et al., 2005) produce an enzyme with cellobiohydrolase activity, capable of processing crystalline cellulose. Brown-rot fungi usually do not produce ligninolytic enzymes, although the

Abbreviations: ABTS, 2,2′-Azino-bis-[3-ethyltiazoline-6-sulfonate]; ASE, acetone soluble extractives; CMCase, carboxymethyl cellulase (endoglucanase); FPA, filter paper activity (total cellulase activity); HTM, hydrothermal modification; MC, moisture content; MnP, manganese-dependent peroxidase; SF, submerged fermentation; SSF, solid-state fermentation; WB, wheat bran; WL, weight loss; 160/3, HTM at 160 °C for 3 h; 170/1, HTM at 170 °C for 1 h.

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production of laccase by *Postia placenta* (Wei et al., 2010) and *Coniophora puteana* (Lee et al., 2004) has been reported earlier.

Wood biodeterioration has been studied not only as an ecological process but also in the context of biopulping (Aguiar and Ferraz, 2008), biorefinery (Ray et al., 2010; Aguiar et al., 2013) and the development of environmentally friendly wood preservation processes. There are several methods to improve the material properties, including decay resistance. Those can be divided into three groups: (hydro) thermal treatments, chemical modification (Sander and Koch, 2001), and impregnation with low molecular weight monomeric compounds (Hill, 2006). Controlled heat treatment of wood at relatively high temperatures ranging from 150 °C to 260 °C is an effective and environmentally friendly method in comparison to chemical modification (Tuong and Li, 2010). Currently, different wood heat treatment processes are available. Thermal treatment of wood is based on the chemical modification of the cell wall components through autocatalytic reactions induced by heat and water (Dietrichs et al., 1978).

An important effect achieved by the thermal treatment of wood is reduced hygroscopicity, and subsequently, increased dimensional stability (Burmester, 1973; Hillis, 1984; Zaman et al., 2000) and improved durability (Kamdem et al., 2002; Boonstra et al., 2007). Šušteršic et al. (2010) and Metsä-Kortelainen (2011) have observed that the decay resistance of different wood species strongly correlates with the severity of the thermal treatment. According to the literature (Kamdem et al., 2002; Hakkou et al., 2006), the reasons for improved durability of HTM wood include: (1) hydrophobic character of wood, which is not favorable to the growth of fungi; (2) generation of new extractives during heat treatment, which are toxic to fungi; (3) modification of wood polymers, leading to the non-recognition of the latter by enzymes; and (4) degradation of hemicelluloses, which is important nutrition for fungi.

The previous studies on the decay resistance of modified wood are almost exclusively based on standard test procedures, which are limited to the determination of weight loss (WL). Scarce information has been obtained on the lignocellulolytic enzyme production in modified wood (Lekounougou et al., 2009; Alfredsen and Fossdal, 2010; Schmöllerl et al., 2011; Elisashvili et al., 2012).

The aim of this study was to evaluate the lignocellulolytic enzyme activity of the white-rot fungus *Trametes versicolor* and the brown-rot fungus *C. puteana*, which are common fungi associated with the wood decay in Europe. These are also obligatory fungi used in the European Standard EN 113 (2000) and the Technical Specification CEN/TS 15083-1 (2005).

Initially, the fungal enzymatic potential was assessed in submerged- (SF) and solid-state fermentations (SSF) using wheat bran (WB) as a well-suited growth substrate, promoting the lignocellulolytic enzyme production. Although SSF has several disadvantages, including mass- and heat-transfer limitations, previous studies prove that SSF, compared to SF, has several advantages: higher enzyme yields, simpler techniques, etc. (Pandey et al., 1999). In SSF, the fungi grow under conditions close to their natural habitat. This may allow them to produce certain enzymes and metabolites, which usually would not be produced or would only be produced at a low yield in SF.

Then the enzymatic activities were compared in colonization of native and hydrothermally modified (HTM) hardwood blocks. Moreover, the profiles of acetone soluble extractives (ASE), and the cellulose content in control and HTM wood blocks were established during the colonization by *C. puteana* to correlate the extent of wood transformation with the levels of extracellular enzymes produced during the fungal growth.

2. Materials and methods

2.1. Fungi and inocula preparation

The white-rot fungus *T. versicolor* (L.) Lloyd (CTB 863A) and the brown-rot fungus *C. puteana* (Schum.:Fr.) Karst. (BAM Ebw.15) were maintained in Petri dishes on a medium containing 5% malt extract concentrate and 2% agar (Fluka, Sigma–Aldrich), pH 6.0. Fungal inocula (concentration 26–31 mg) were prepared by growing the strains on a rotary shaker (Infors, Switzerland) at 150 rpm and 27 °C in 250-ml flasks filled with 100 ml of the standard medium, containing glucose (15.0 g l^{-1}), peptone (3.0 g l^{-1}), yeast extract (3.0 g l^{-1}), NaH_2PO_4 (0.8 g l^{-1}), $K_2HPO_4 \cdot 3H_2O$ (0.4 g l^{-1}), $MgSO_4 \cdot 7H_2O$ (0.5 g l^{-1}); initial pH 6.0. After 5 days of cultivation, mycelial pellets were homogenized using a Waring laboratory blender.

2.2. Cultivation conditions

SF of selected fungi was carried out on a rotary shaker at 150 rpm and 27 °C in 250-ml conical flasks filled with 50 ml of the above-mentioned medium, containing glycerol (15.0 g l^{-1}) or WB (40.0 g l^{-1}) instead of glucose. Initial pH was adjusted to 6.0. After sterilization at 121 °C for 35 min, the flasks were inoculated with 7 ml of homogenized mycelium. At time intervals of 7, 14, and 21 days, samples (2 ml) were taken, centrifuged at 4000 rpm for 12 min, and the filtrates were used for the target enzyme activity determination.

The SSF of WB was carried out under stationary conditions at 22 °C and 70% relative humidity (RH) in 100-ml conical flasks, containing 4 g of the substrate moistened with 12 ml of the above-mentioned medium without glucose. Flasks were sterilized at 121 °C for 35 min and inoculated with 5 ml of the homogenized mycelium. After 7, 14, and 21 days of cultivation, the extracellular enzymes were extracted twice from the fermented products with 25 ml of distilled water (total volume 50 ml). The extracts were centrifuged at 4000 rpm for 12 min, and the filtrates were used for the enzyme activity determination.

2.3. Wood decay tests

Birch (*Betula* spp.), aspen (*Populus tremula*) and grey alder (*Alnus incana*) wood was hydrothermally modified in a laboratory multifunctional experimental device in the water vapor medium at 160 °C for 3 h and 170 °C for 1 h as described before (Andersons et al., 2010; Grinins et al. 2013).

HTM and untreated control wood blocks (20 × 20 × 5 mm) were exposed to *C. puteana* and *T. versicolor* on a medium containing 5% malt extract concentrate and 2% agar in Petri dishes. The test procedures of CEN/TS 15083-1 (2005) were followed. Sterile wood blocks with the largest face (cross section) were aseptically placed on 3-mm stainless steel supports and incubated at 22 °C and 70% RH for 10, 20 and 42 days. Three replicates for each wood species, treatment regime and test period were taken.

Subsequent to cultivation, the blocks were removed from the Petri dishes, crushed in smaller pieces and milled to a particle size ~1 mm. For the enzyme extraction, milled samples were treated twice (7 ml and 8 ml) with distilled water for 15 min. Then the samples were centrifuged at 4000 rpm for 12 min, and supernatants were used for determination of enzyme activities. For WL measurements, the blocks after cultivation were removed from the Petri dishes, brushed free of mycelium and oven dried at 103 °C for 5 h. WL (%) was calculated from the dry weight before and after the test. The wood moisture content (MC, %) was calculated from the wet weight and the dry weight after the test.