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Biodegradation of treated softwood and hardwood species by brown rot fungi

Anna Janberga[1]*, Ilze Irbe[1], Vladimirs Biziks[1], Nina Kurnosova[1], Mikheil D. Asatiani[2], Bruno Andersons[1], Ingeborga Andersone[1]

[1]Latvian State Institute of Wood Chemistry, Dzerbenes 27, Riga LV–1006, Latvia
[2]Durmishidze Institute of Biochemistry and Biotechnology, Georgian Agrarian University, 10 km Agmasheneblis kheivani, Tbilisi 0159, Georgia

*Corresponding author, E-mail: aj07019@lanet.lv

Abstract

The aim of this study was to investigate enzymatic activity and biodegradation capacity of treated wood by three brown rot fungi. For measurement of enzymatic activity, the brown rot fungi *Coniophora puteana, Postia placenta* and *Gloeophyllum trabeum* were grown in solid state fermentation of wheat bran-containing medium. The highest enzymatic activity of all enzymes, i.e. 8.3 U mL^{-1}, was detected for *C. puteana*-produced xylanase. The highest activity of endoglucanase, i.e. 4.4 U mL^{-1}, was detected for *P. placenta*. Total cellulolytic activity showed the lowest value (0.1 U mL^{-1}) for all the fungi under study. To improve the durability of softwoods, they were impregnated with wood preservatives Celcure AC 500 and Dikants and hydrothermally modified in two regimes, 150 °C for 3 h and 160 °C for 1 h. Hardwoods were hydrothermally modified at 160 °C for 1 h. The efficiency against softwood degradation by *C. puteana, G. trabeum* and *P. placenta* and that against hardwood degradation by *C. puteana* were determined according to the standards CEN/TS 15083-1 and EN 84. Heat treatment was more effective than chemical treatment. Hydrothermal modification by the same conditions (temperature/time) was more effective for softwoods than hardwoods.

Key words: brown rot fungi, endoglucanase, enzymatic activity, wood preservatives, wood durability, xylanase.
Abbreviations: HTM, hydrothermal modification; SSF, solid state fermentation.

Introduction

Wood rotting fungi are usually divided into three groups: white rot, soft rot and brown rot (Eriksson 1990). In the temperate climate zone, 80% of wood construction damage is caused by brown rot (Green, Highley 1995; Irbe et al. 2001). Brown rot fungi degrade wood cellulose and hemicelluloses, whereas lignin is modified. Brown rot fungi degrade cellulose and hemicelluloses with enzymes and hydroxyl radicals (Jin et al. 1990). The following extracellular enzymes are involved in cellulose degradation: endo-1,4-β-glucanase (EC 3.2.1.4), exo-1,4-β-glucanase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21). Most of brown rot fungi do not produce exo-1,4-β-glucanase, except for some strains of *Coniophora puteana* (Schmidhalter, Canevascini 1993). Hemicellulases are classified according to their substrate specificity. The main extracellular enzymes which are involved in hemicellulose degradation are endo-1,4-β-xylanase (EC 3.2.1.8), endo-1,4-β-mannosidase (EC 3.2.1.78), β-D-xylosidase (EC 3.2.1.37) and β-D- mannosidase (EC 3.2.1.25) (Eriksson et al. 1990). The information available on fungal cellulases and hemicellulases is based mostly on studies of the filamentous fungus *Trichoderma reesei* and the white rot fungus *Phanerochaete chrysosporium*.

There are several methods of how to improve wood durability against brown rot fungi, such as chemical impregnation, thermal modification, and chemical modification. Heat treatment has been developed as an alternative to increase service life for wood materials without the use of toxic chemicals as in chemical impregnation (Rowell 2010). There are several hypotheses about the reasons why thermally modified wood has improved durability against brown rot fungi. One hypothesis that might be associated with the fungi enzymatic system is that structure of wood components (cellulose, hemicelluloses and lignin) is changed and enzymes do not recognize the substrate (Rowell et al. 2009).

Knowledge about the extracellular enzyme system of brown rot fungi would help to better understand the mechanisms of wood degradation. The understanding of wood biodegradation would be a key factor for improvement or invention of new methods to increase wood durability. The objectives of this study were (i) to compare the enzymatic activity of three brown rot fungi and (ii) to investigate two different strategies on how to improve wood durability against biodegradation, namely by impregnation and hydrothermal modification (HTM).

Materials and methods

Fungi strains and inoculum preparation

The wood destroying basidiomycetes *Coniophora puteana* BAM Ebw. 15, *Gloeophyllum trabeum* BAM Ebw. 109 and *Postia placenta* FPRL 280 (from Bundesanstalt für Materialforschung und –prüfung, Germany) were used. Fungal strains were maintained on 5% malt extract concentrate (w/w) and 2% agar (w/w) media (European Standard CENTS 15083-1) at 5 °C. Inoculate was grown in 200 mL flasks with 100 mL of synthetic medium containing 15.0 g L^{-1} glucose, 3.0 g L^{-1} peptone, 3.0 g L^{-1} yeast extract, 0.8 g L^{-1} NaH_2PO_4, 0.4 g L^{-1} K_2HPO_4 $3H_2O$, and 0.5 g L^{-1} $MgSO_4$ $7H_2O$ (Elisashvili et al. 2012). The medium pH was adjusted to 6.0 (Knick, Germany). Medium was sterilized at 121 °C for 20 min (Getinge, Sweden). The fungi were cultivated on a rotary shaker (Infors AG, Switzerland) at 150 rpm for 5 days at 27 °C. All chemicals used were from Sigma–Aldrich, if not indicated differently.

Fungi cultivation in solid state fermentation

The source of lignocellulose in solid state fermentation (SSF) was wheat bran. SSF was performed at 22 °C and 70% RH in 100 mL Erlenmeyer flasks containing 4 g wheat bran, 12 mL synthetic medium (without glucose) and 5 mL of inoculate. The initial pH of medium was adjusted to 6.0. The medium was sterilized at 121 °C for 30 min. Inoculate was added to medium after sterilization. The fungi in SSF were cultivated in triplicate for each period. The extracellular hydrolases after 7, 14, and 21 days of cultivation were extracted twice from total biomass with 25 mL of distilled water (Elisashvili et al. 2012). Homogenization was performed manually. Homogenates were filtered through nylon cloth and centrifuged for 12 min at 4000 rpm (Sigma, Germany). The supernatants were stored at 4 °C until measurement of enzymatic activity.

Measurement of enzymatic activity

Enzymatic activity of xylanase and endoglucanase, and total cellulolytic activity were measured according to IUPAC (International Union of Pure and Applied Chemistry) recommendations. Three replicates for each enzyme activity were used.

Endoglucanase activity was measured using low viscosity carboxymethyl cellulose (2% w/v) (Sigma Aldrich, USA) as a substrate. The reaction mixture contained 100 µL of carboxymethyl cellulose (2%) diluted in 50 mM citrate buffer (pH 5.0) and100 µL of the supernatant. The reaction mixture was incubated at 50 °C (Biosan, Latvia) for 10 min (Ghose 1987).

Xylanase activity was measured using birch xylan (1% w/v) (Roth, Germany) as a substrate. The reaction mixture contained 630 µL of xylan diluted in 50 mM citrate buffer (pH 5.0) and 70 µL of the supernatant. The reaction mixture was incubated at 50 °C for 10 min (Bailey et al. 1992).

Total cellulolytic activity was measured using Whatman filter paper No. 1 (Whatman, England) as a substrate (Ghose 1987). The reaction mixture contained 1 mL 50 mM citrate buffer, 500 µL supernatant and 50 mg filter paper. The reaction mixture was incubated at 50 °C for 1 h.

Reducing sugars were determined using the dinitrosalicylic acid reagent method for all enzyme measurements (Miller 1959).

One unit of enzymatic activity was defined as the amount of enzyme that releases 1 µmol reducing sugar (glucose or xylose) in 1 min per mL filtrate (Ghose 1987; Bailey 1992).

Chemical impregnation of wood

Impregnation was carried out on pine (*Pinus sylvestris* L.) and spruce [*Picea abies* (L.) H.Karst.] wood. The wood was dipped into a preservative for no less than 6 h. Vacuum treatment was done using industrial equipment (15 to 30 min vacuum, 45 to 60 min pressure 10 to 12 MPa, 10 to 15 min vacuum).

Impregnation was done with one of the two preservatives, Celcure AC 500 or Dikants. Celcure AC 500 is composed of 16.53% copper hydroxycarbonate, benzalkonium chloride and boric acid. Dikants is composed of chromium, fluorine and boron compounds (chromium compounds ~20%). After impregnation, all wood samples were coated with linseed oil.

Wood hydrothermal modification

The wood used for HTM was grey alder [*Alnus incana* (L.) Moench], aspen (*Populus tremula* L.), birch (*Betula pendula* Roth), pine (*Pinus sylvestris* L.) and spruce [*Picea abies* (L.) H. Karst.]. Treatment was carried out in a multifunctional wood modification pilot device (WTT, Denmark). Wood was hydrothermally modified in water vapour medium. For 1 g of oven dry wood, 0.67 mL of water was pumped into the device. HTM was done in two regimes, namely, 150 °C for 3 h and 160 °C for 1 h.

Decay test

The test was performed according to European Standards CEN/TS 15083-1 and EN 84. The test procedure of CEN/TS 15083-1 was slightly modified. The wood samples (2 × 2 × 0.5 cm), two treated and two untreated, were placed into a Petri dish with 5% malt extract concentrate (w/w) and 2% agar (w/w) media (European Standard CENTS 15083-1) for 6 weeks. Four replicates for each fungus and each preservation strategy were tested.

Statistical analysis

Means and standard deviations were calculated. The Student *t*-test was used to test differences among fungi and hardwoods and softwoods. $P < 0.05$ was considered statistically significant.

Results

In order to determine capacity of extracellular enzyme production brown rot fungi *C. puteana, G. trabeum* and *P. placenta* were grown in SSF using wheat bran as a lignocellulose substrate. SSF is considered as the most appropriate method for cultivation of basidiomycetes (including brown rot fungi), because they grow under conditions close to these in their natural habitats (Pandey et al. 1999). Extracellular enzyme activities and pH values of brown rot fungi cultivated in SSF are shown in Table 1. The highest enzymatic activity of all enzymes (8.3 U mL^{-1}) was detected for *C. puteana*-produced xylanase. The highest activity of endoglucanase, i.e. 4.4 U mL^{-1}, was detected for *P. placenta*. Total cellulolytic activity showed the lowest activity (0.1 U mL^{-1}) of all three measured enzymes. The maximum of endoglucanase activity was on the 7th day, whereas for xylanase on the 21st day. pH values strongly varied from very acidic, i.e. 2.2 (*P. placenta* after 14 days in SSF) to almost neutral, i.e. 6.6 (*G. trabeum* after 21 days).

The natural durability against rot fungi of grey alder, aspen, birch, pine and spruce wood is classified as non-durable (British Department of Environment 1977; LVS EN 350-2:2000). To determine the efficiency of treatment methods for wood durability, tests were conducted in accordance with European standards CEN/TS 15083-1 and EN 84. According to CEN/TS 15083-1, the first durability class (very durable wood) corresponds to mass loss ≤ 5%. Decay test results for hydrothermally modified and chemically impregnated softwoods are shown in Table 2. For validity of results, the mass loss of control specimens should be greater than 20%; in our case the criteria were met (data not shown). The results for unleached wood (CEN/TS 15083-1) showed that all preservation strategies reached the first durability class, except in the case of impregnation with Celcure AC 500 (amount of the chemical preservative 1.1 kg m^{-3}) by dipping for spruce, where the mass loss was 5.1%. The best results (mass loss 1%) according to CEN/TS 15083-1 were obtained for Celcure AC 500-vacuum treated pine (amount of the chemical preservative 3.3 kg m^{-3}).

Results for leached samples showed that, in almost all cases when using dipping, the durability against fungi was unsatisfactory, except for spruce treated with Celcure AC 500 (amount of the chemical preservative 1.3 kg m^{-3}), where the mass loss was 1% after testing with *P. placenta*. Results of the tests with leaching (EN 84) showed that the first durability class was reached for vacuum-treated pine with Celcure AC 500, where the mass loss after *G. trabeum* and *P. placenta* was 0.3%. The results of the tests according to EN 84 indicated that none of the chemical preservatives after leaching was effective against *C. puteana*, and mass loss was 26.0 to 41.5 %. The difference of mass loss was statistically significant ($P < 0.05$) for *C. puteana* and *P. placenta*. The difference of mass loss was not statistically significant for *C. puteana* and *G. trabeum*, and *G. trabeum* and *P. placenta* ($P < 0.05$).

For pine, effective protection was gained in both HTM regimes. After leaching, for spruce the first durability class was not reached against *G. trabeum* and *P. placenta* in the modification regime 150 °C for 3 h. The spruce modified at 160 °C for 1 h had lower mass loss than that modified in the modification regime 150 °C for 3 h. For spruce modified at 160 °C for 1 h after leaching, the first durability class was not reached against *G. trabeum.* After leaching, the durability against *G. trabeum* for HTM pine improved. For HTM spruce, the durability against G. trabeum after leaching declined. The same situation was observed for impregnated samples.

Decay tests were done for untreated and hydrothermally modified hardwood. The first durability class for HTM hardwoods at 160 °C for 1 h was not reached. To gain the first durability class for hardwoods, the modification regime must be changed, i.e. modification at a higher temperature or for a longer period of time should be carried out. According to CEN/TS 15083-1, the mass loss of HTM hardwood at 160 °C for 1 h was 7.1, 7.1 and 13.3% for aspen, birch and grey alder, respectively, but after leaching according to EN 84, mass loss was 17.2, 20.0 and 26.2% for aspen, birch and grey alder, respectively (Table 3).

For untreated grey alder, the mass loss against *C.*

Table 1. Enzymatic activity of *Coniophora puteana, Postia placenta* and *Gloeophyllum trabeum* after 7, 14 and 21 days in SSF of wheat bran

Time	Species	pH	Endoglucanase (U mL^{-1})	Xylanase (U mL^{-1})	Total cellulolytic activity (U mL^{-1})
7 days	*C. puteana*	3.5	1.5 ± 0.23	1.9 ± 0.05	0.1 ± 0.01
	P. placenta	2.3	4.4 ± 0.20	0.6 ± 0.04	0.1 ± 0.01
	G. trabeum	4.2	1.0 ± 0.02	1.3 ± 0.05	0.1 ± 0.00
14 days	*C. puteana*	4.6	0.9 ± 0.02	4.5 ± 0.44	0.1 ± 0.00
	P. placenta	2.2	1.7 ± 0.05	0.6 ± 0.13	0.1 ± 0.01
	G. trabeum	6.1	0.9 ± 0.01	1.7 ± 0.19	0.1 ± 0.00
21 days	*C. puteana*	6.0	0.7 ± 0.03	8.3 ± 0.49	0.1 ± 0.00
	P. placenta	2.5	1.5 ± 0.05	0.8 ± 0.13	0.1 ± 0.00
	G. trabeum	6.6	0.9 ± 0.04	3.1 ± 0.35	0.1 ± 0.00

Table 2. Mass loss (%) of spruce and pine in the tests CEN/TS 15083-1 and EN 84 with *Gloeophyllum trabeum, Coniophora puteana* and *Poria placenta*

Preservation strategy	Species	CEN/TS 15083-1	CEN/TS 15083-1 and EN 84		
		G. trabeum	*C. puteana*	*G. trabeum*	*P. placenta*
Dikants, dipping (retention 2.0 kg m^{-3})	Spruce	1.2 ± 0.3	41.5 ± 6.6	25.9 ± 3.3	13.0 ± 4.1
Celcure AC 500, dipping (retention 1.1 kg m^{-3})	Spruce	5.1 ± 7.0	37.4 ± 5.6	10.5 ± 1.3	–
Celcure AC 500, dipping (retention 1.3 kg m^{-3})	Spruce	4.9 ± 5.0	38.0 ± 12.2	14.3 ± 14.1	1.0 ± 1.5
Celcure AC 500, vacuum treatment (retention 3.3 kg m^{-3})	Pine	1.0 ± 0.1	26.0 ± 7.8	0.3 ± 0.5	0.3 ± 0.5
Celcure AC 500, vacuum treatment (retention 5.0 kg m^{-3})	Pine	1.5 ± 0.3	32.5 ± 6.7	0.3 ± 0.3	0.3 ± 0.3
HTM 150 °C 3 h	Pine	3.7 ± 2.0	0.5 ± 1.1	1.0 ± 0.9	3.0 ± 3.3
HTM 160 °C 1 h	Pine	2.1 ± 0.4	0.2 ± 0.5	0.1 ± 0.2	0.2 ± 0.3
HTM 150 ° C 3 h	Spruce	1.0 ± 0.8	2.3 ± 3.8	14.1 ± 3.4	7.6 ± 4.1
HTM 160 °C 1 h	Spruce	1.5 ± 1.0	0.0 ± 0.0	5.5 ± 2.5	1.3 ± 1.5

puteana after leaching increased from 31.9 to 40.6%. For untreated aspen and birch, the mass loss against *C. puteana* after leaching declined from 52.0 to 43.4% and from 49.9 to 44.7% for aspen and birch, respectively (Table 3).

The results showed that HTM at 160 °C for 1 h gave better durability against *C. puteana* for softwoods than hardwoods. The difference of mass loss between softwoods and hardwoods was statistically significant ($P < 0.05$).

Discussion

Extracellular enzymatic activity of brown rot fungi depends on many factors like pH, temperature, substrate, aeration and cultivation method (Keilich et al. 1970; Tholudur et al. 1999). The day of maximum enzymatic activity differs in various studies. In our case the maximum activity for endoglucanase and xylanase was observed in different days (7 and 21 respectively). Elisashvili et al. (2012) detected maximum activity of endoglucanase and xylanase on day 21 in SSF.

The pH range in which brown rot fungi has enzymatic activity is very broad, from 2.1 to 7.5 (Herr et al. 1978; Valášková, Baldrian 2006). Initial pH of media in our study was 6.0. The decrease of pH after 7 days of cultivation might have been caused by the production of organic acids (oxalate, citric acid). The decrease of pH in the case of *G. trabeum* was less than that for *C. puteana* and *P. placenta*, because it does not accumulate oxalate (Green et al. 1991).

C. puteana has pH optimum 3.5 to 4.0 for cellulase production, *G. trabeum* 4.4 and *P. placenta* 4.0. The pH optimum differs in various conditions. In the study conducted by Highley (1983), *G. trabeum* had optimum pH 3.0 for endocellulase production.

In submerged fermentation, *G. trabeum* had optimal pH for endoglucanase activity 4.4, but at pH 7.5 the fungi showed only 15% of its maximum activity (Herr et al. 1978). In our case, the activity was almost the same at pH 4.2 and 6.6. The optimum pH for *G. trabeum* xylanase was 4.0, but at pH 7.0 the relative activity was 40%. (Ritschkoff et al. 1994). We observed higher xylanase activity at pH 6.6 than at pH 4.2.

Some studies indicated that high enzymatic activity does not necessarily mean that mass loss of a specific component will be high (Machuca, Ferraz 2001). Interestingly, in our research *C. puteana* showed the highest mass loss of chemically impregnated wood and also xylanase activity. It is known that hemicelluloses (including xylan) are wood structural components that are degraded first by brown rot fungi (Tjerdsmaa et al. 1998). The reason why the mass loss of HTM wood for *C. puteana* was not higher than for the other two brown rot fungi might be attributed to HTM processes where hemicelluloses are degraded at lower temperatures than cellulose and lignin (Tjerdsmaa et al. 1998).

The mass loss changes between unleached and leached wood were various for different wood species. For

Table 3. Mass loss (%) of grey alder, aspen and birch wood in the tests CEN/TS 15083-1 and EN 84 with *Coniophora puteana*

Species	Preservation strategy	CEN/TS 15083-1	CEN/TS 15083-1 and EN 84
Grey alder	Untreated	31.9 ± 10.0	40.6 ± 3.2
	HTM 160 °C 1 h	13.3 ± 8.2	26.2 ± 10.6
Aspen	Untreated	52.0 ± 5.2	43.4 ± 3.1
	HTM 160 °C 1 h	7.1 ± 3.9	17.2 ± 9.9
Aspen	Untreated	49.9 ± 2.7	44.7 ± 1.8
	HTM 160 °C 1 h	7.1 ± 0.8	20.0 ± 0.6

chemically preserved spruce, the mass loss increase can be explained by partial leaching of the chemical preservative components. The decay activity of *G. trabeum* for HTM spruce was probably stimulated by leaching of spruce extractives, which inhibited fungal growth. For HTM pine, durability improved, which can be explained by the washing away of easily taken up degradation products such as sugars (Biziks et al 2009). The decay activity of *C. puteana* for HTM hardwoods was probably stimulated by leaching of low molecular compounds, which inhibited the growth of fungi (Biziks 2011).

There are few studies in which hardwoods and softwoods are modified in the same process conditions and also in which the durability against brown rot fungi is determined. For hardwoods, the mass loss after thermo modification is higher than for softwoods, because of their differences in hemicelluloses (Fengel, Wegener 1989; Rowell et al. 2009). It is widely accepted that the higher mass loss after thermo modification indicates better resistance against biodegradation. Thermally modified beech showed better resistance against *P. placenta* than thermally modified maritime pine (Weiland, Guyonnet 2003). After our HTM treatment, softwoods showed higher resistance against *C. puteana* than hardwoods. Further research should be conducted to obtain additional data about enzymatic activity on different lignocellulosic substrates and wood treatments.

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