Enzymatic Hydrolysis of Furfurylated Scots Pine Sapwood (*Pinus sylvestris*, L.)

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ABSTRACT

The effect of moderate furfurylation of Scots pine sapwood upon the release of wood carbohydrates during enzyme-catalyzed hydrolysis has been investigated. The work was initiated to better understand the mechanisms in wood furfurylation that render a material with improved fungal decay resistance; thought mainly to be a result of reduced equilibrium moisture content by cell wall bulking. The results show that moderate furfurylation (weight percent gain, WPG 20-25%) reduces the release of carbohydrates with approximately 55-60% in total when a specified mix of commercially available enzyme preparations is applied to a liquid suspension of milled wood. A dose-response relationship was derived based on furfurylation intensity. Comparison to a decay trial indicate that the described set-up and analytical method based on high-performance anion exchange chromatograhpy (HPAEC), may be a viable tool for further investigations regarding decay protection mechanisms in modified wood.

INTRODUCTION

Two wood modification methods, furfurylation and acetylation, both improving the biological durability of wood have recently been commercialized. Bulking of the wood cell walls by covalently bonded adduct is believed to be the main mode of action in acetylation (Hill *et al.* 2007). This bulking lowers the equilibrium moisture content (EMC) up to 50% (Hill and Jones 1996, Papadopoulos and Hill 2003) which in turn confers decay protection (Hill *et al.* 2007). Furfurylation is mainly thought to provide moisture exclusion and thus decay protection by steric hindrance or physical bulking by an *in-situ* generated furan polymer in the wood cell walls. The actual mechanisms involved are not well understood (Lande 2008). Lowering of the EMC by 30-50% has been observed (Epmeier *et al.* 2004, 2007). Though both acetylation and furfurylation bulk the cell walls, they require different levels of weight gain to attain decay resistance. Acetylation to 18-23% WPG seems to offer good protection both for above and in-ground applications (Hill 2006, Larsson Brelid *et al.* 2000), while furfurylated wood in ground contact must be treated to approximately 40-50% WPG to render sufficient decay protection (Lande 2008). One explanation to this dissimilarity might be differences in the protection mechanism other than exclusion of moisture. One such difference could be the accessibility of the wood towards the fungal enzymes responsible for degradation. Microbial degradation of wood is dependent upon the accessibility of the lignin, hemicelluloses and cellulose to the fungal enzymes. If modification of wood not only excludes water from the wood structure, but also reduces

the accessibility of the wood components it may offer a very effective protection. Likewise; an evaluation of the accessibility of the wood components towards fungal enzymes may enable a better understanding of how the wood is modified. Degradation of hemicelluloses has been suggested to be one of the most important initial stages in brown-rot decay (Green III and Highley 1997). Consequently, a wood modification method that reduces the overall accessibility of hemicelluloses (and cellulose) for enzymatic hydrolysis will have a protective effect. Many basidiomycetes also secrete extracellular oxidative enzymes (oxidoreductases and peroxidases) which are involved in the degradation of cell wall components, e.g. lignin removal by white-rot fungi (Martinez *et al.* 2005). Altering the oxidation potential of lignin could similarly offer decay protection.

Ritschkoff *et al.* (1999) reported on the release of carbohydrates from resin bulked wood during hydrolysis using an isolated enzyme preparation. They found that there was a significantly reduced hydrolyzability of carbohydrates in these modified products compared to untreated wood. Based on this and other assays they concluded that the protective mechanism was a result of inhibition of the catalytic action of the enzymes and/or a reduction in the extracellular release of enzymes by fungi growing on modified wood substrates (*op. cit.*). So-called non-recognition of modified wood substrates by enzymes is often suggested to play a role in decay protection, see e.g. the overview on heat treated timber by Rapp *et al.* (2008), but data to support whether it should have an effect is limited. Decay fungi use a variety of enzymes to catalyze their breakdown reactions. Two main classes are as indicated above; hydrolytic and oxidative enzymes. The first group is the subject of this paper. A range of enzymes are needed to degrade cellulose and hemicelluloses due to a number of different linkages being involved in their respective structures (Sørensen *et al.* 2003). Before the hydrolytic enzymes can access the carbohydrates, cell walls must be opened up, since enzymes are too large to penetrate openings in sound solid wood (Evans *et al.* 1994; Green III and Highley 1997). The systems responsible for this are subject to much research and are commonly referred to as low molecular weight diffusible/degradative agents or reactive oxygen species, see *e.g*. Hammel *et al.* (2002). The overall aim of the present work was to study how moderate furfurylation of Scots pine sapwood (WPGs < 30%) affects hydrolytic enzyme action on wood. An enzymatic assay capable of quantifying the accessibility and hydrolysis of wood carbohydrates is developed and presented.

MATERIALS AND METHODS

Wood material

Defect free, straight grained Scots pine (*Pinus sylvestris*, L.) sapwood was sawn and planed to give 100 blocks nominally measuring 15 x 25 x 50 mm^3 at 20 °C, 65% RH. The mean basic density in oven-dry condition ($r_{0,0}$) was 471 kg/m^3 with a standard deviation of 33 kg/m^3. The wood was pre-dried at 103 °C for 16 hours before impregnation and weighed (W_1) after having settled to ambient temperature in a desiccator.

Wood modification

Wood furfurylation is a process consisting of a vacuum pressure impregnation of permeable wood with initiated furfuryl alcohol followed by curing and drying which results in the formation of a furan polymer inside the wood cell walls. Diluted solutions of furfuryl alcohol are normally employed which necessitates solvent removal before

curing. Chemicals used in the modification process were: Furfuryl alcohol (FA) (>98%, TransFurans Chemicals), citric acid (CA) (99%, water-free, Sigma-Aldrich), maleic anhydride (MA) (>99%, KEBOLab), ethanol (EtOH)(96%, VWR International) and de-ionized water. CA functions as catalyst to the polymerization reaction since FA resinifies in acid media (Rathi and Chanda 1974). The MA component acts both as part of the catalyst system as well as a coupling agent to enhance the supposed bonding to wood (Schneider and Phillips 2004). Ethanol addition keeps pH neutral during the impregnation phase and thereby prevents untimely polymerization (Westin 2004). Different impregnation solutions were prepared to reach a range of treatment intensities. CA was added in amounts of 0.25 to 4.0 wt-% of total solution; MA was added in amounts of 0.13 to 2.0 wt-% of total solution. The modification treatment consisted of five consecutive steps:

1) Wet vacuum: 0.1 bar for 0.5 hour
2) Pressure: 11 bar for 2 hours
3) Evaporation of solvent: 20→40 °C (temperature ramp) for 4 hours at 0.2 bar
4) Curing: 103 °C for 16 hours wrapped in aluminium foil
5) Final drying to evaporate condensation water and unreacted monomer

After modification treatment, samples were leached in de-ionized water according to EN84 (CEN 1997a) and re-weighed after oven drying at 103 °C for 16 hours (W_2) following the procedure described above. Leaching was performed in order to remove easily soluble constituents in untreated wood and unreacted, leachable monomers and oligomers in furfurylated wood. The treatment intensity was calculated as weight percent gain (WPG) according to Eqn. 1:

$$\mathrm{WPG}\,(\%) = \frac{W_2 - W_1}{W_1} \times 100\% \qquad (1)$$

Sample dimensions were obtained before and after modification in order to determine gross swelling. Acetylated radiata pine was obtained commercially from Titanwood, Holland. 50 mm sections were cut from the middle of 15 boards measuring 22 x 150 x 2000 mm^3, converted to blocks sized 15 x 22 x 50 mm^3. According to the supplier, samples were estimated to have an approximate acetyl content of 20% resulting in a WPG of 23 % based on extrapolation of the formula given by Stefke *et al*. (2008).

Enzymes

Commercial fungal enzyme preparations from *Trichoderma reesei*, (Celluclast 1.5L), *Humicola insolens* (Ultraflo L), and *Aspergillus niger* (Gammanase 1.0L) were obtained from Novozymes A/S, Bagsværd, Denmark. Celluclast and Ultraflo are fermentation broths containing a wide range of hydrolytic enzymes acting on cellulose, hemicelluloses and pectins. A mixture of these two enzymes has previously been shown to degrade lignocellulosic carbohydrates effectively (Sørensen *et al*. 2003, 2005) thus efficiently simulating fungal degradation. However, both products lack mannanase activity and Gammanase was applied to hydrolyze the mannose component as well.

Enzymatic hydrolysis

Enzymatic release of carbohydrates was performed on water-leached blocks of furfurylated, acetylated and untreated wood. Prior to hydrolysis the blocks were milled to a particle size ≤ 2 mm and subsequently transported to 100 mL blue cap bottles. Each

sample of substrate was prepared so that it contained 2.0 g wood dry matter (DM). A 1:1:1 mix by weight of Celluclast 1.5 L, Ultraflo L and Gammanse 1.0L was added in a 50 mM citrate buffer (pH 5.0) using a 5 wt-% enzyme/wood DM ratio to obtain a total DM content in the system of 4%. Samples were incubated at 50 °C for 24 or 48 hours (rotation: 150 rpm). The enzymatic hydrolysis experiments were performed in triplicate. After incubation, 2.5 mL of the supernatant hydrolysate was transported to Eppendorf tubes and heated at 100 °C for 5 min to stop enzymatic activity. Prior to analysis, samples were passed through a syringe membrane filter and diluted appropriately in Milli-Q water.

Determination of released carbohydrates

The concentrations of released carbohydrates were determined by High-Performance Anion Exchange Chromatography. Hydrolysates were applied onto a Dionex BioLC system fitted with a Dionex CarboPac PA1 analytical column combined with a Carbo-Pac PA1 precolumn. Monosaccharides were separated isocratically with 2 mM KOH for 32 min. Strongly retained anions were removed from the column by increasing the KOH concentration to 25 mM over a period of 7 min, and after 5 min at 25 mM the column was equilibrated at 2 mM for 10 min. Monosaccharides were detected by a pulsed electrochemical detector in the pulsed amperiometric detection mode. A mixture of arabinose, galactose, glucose, xylose and mannose (each component 5 to 100 mg/L) was used as standard.

Assessment of fungal decay protection

50 blocks of the furfurylated material were tested according to EN113 (CEN 1997b) with *Coniophora puteana* (BAM Ebw. 15) as test fungus in order to compare results to the enzymatic hydrolysis trial.

RESULTS AND DISCUSSION

Wood modification

The WPG range obtained by furfurylation was 7-30% after leaching. The batch was divided into five groups according to WPG and two samples selected at random from each group for hydrolysis.

Enzymatic hydrolysis

The total release of monosaccharides from furfurylated samples (0% WPG [untreated] to 23% WPG) upon treatment with Celluclast 1.5L, Ultraflo L, and Gammanase 1.0L during 24 and 48 hours is shown in figure 1. An acetylated sample is shown as reference. The mass loss of untreated wood due to hydrolysis was, calculated on basis of released carbohydrates, approximately one to two weight percent after 48 hours of incubation.

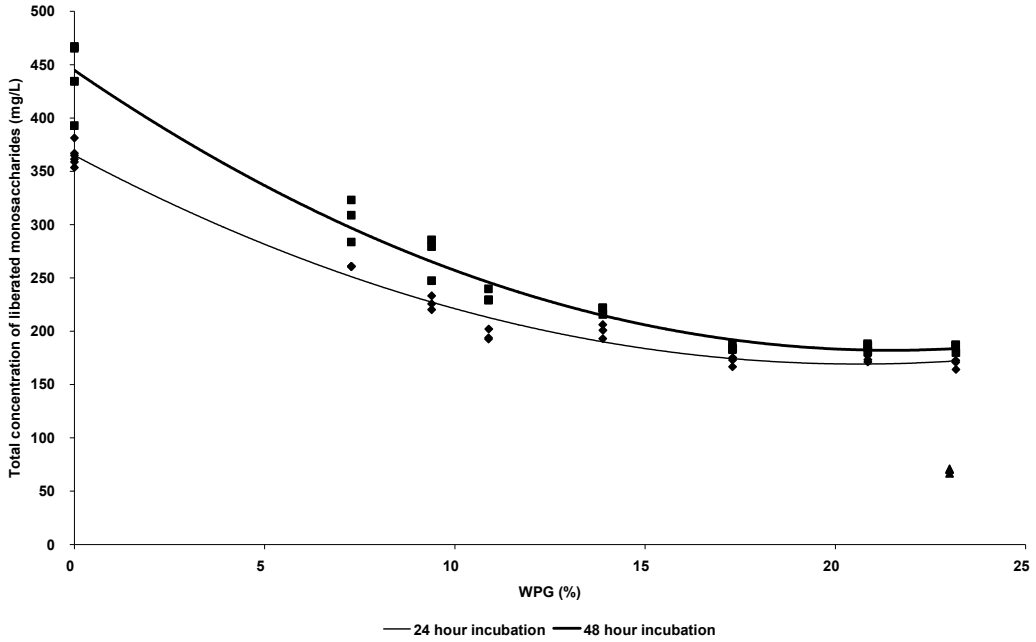

Figure 1: Total concentration of released monosaccharides (mg/L) in hydrolysates from furfurylated Scots pine sapwood as a function of weight percent gain (WPG). Narrow black line (R^2 = 0.98) and diamonds (♦) mark analysis results for furfurylated samples incubated for 24 hours while bold line (R^2 = 0.97) and squares (■) mark results from 48 hours of incubation. For comparison, triangles (▲) mark results for acetylated samples after 48 hours of incubation.

A clear effect of furfurylation is seen even at 7% WPG. Furfurylation resulting in a 20-25% WPG reduces the hydrolytic breakdown of cellulose and hemicelluloses by approximately 55% compared to the untreated control at 24 hours of incubation. In comparison, acetylation within the approximate same range of WPG offered an 80% reduction. Samples incubated for 48 hours released slightly higher amounts of monosaccharides compared to that obtained in a 24 hour reaction. Especially untreated samples release more while the difference between 24 and 48 hours of incubation decreased with increasing WPG by furfurylation, see fig. 1. Acetylated samples also released slightly more during a 48 hour incubation compared to 24 hours. The reduction in total release of carbohydrates at 48 hours of incubation was 60% for furfurylated material (20-25% WPG) and 85% for acetylated material (approximately 23% WPG). Enzymes are large molecules which for a number of the cellulases have specific binding modules. Thus the decrease in carbohydrate release from both types of modified wood can be caused by bulking of the wood cell walls which blocks the access as well as a structural modification which may prevent substrate recognition. However, the observations of released carbohydrates from acetylated and in particular furfurylated wood show that both modified woods are still subject to breakdown by fungal carbohydrases. The release of individual monosaccharides as a function of WPG found a decrease with increasing WPG. Glucose is released to the greatest extent – constituting 64% in mean of the total release (data not shown). The level of glucose release stabilizes at 20-25% WPG by furfurylation. Mannose, xylose and galactose are released in amounts of approximately 10, 5 and 3% of total. The release of mannose seems to have a slower rate compared to the other monosaccharides. For arabinose, the release did not decrease with increasing WPG. We suspect that this may be an artefact related to the furfurylation. The carbohydrate release stabilizes around 20-25% WPG. This corresponds to the point where gross swelling of samples after furfurylation also levels

off (data not shown). This supports that reduced accessibility due to bulking is a main mechanism behind the reduction in carbohydrate release. For acetylated wood the release of all monosaccharides was markedly reduced; also for arabinose. The release of this sugar was reduced to approximately 25% compared to untreated wood and constituted about 12% of total release. Galactose was in mean 7% of total release, glucose 68%, xylose 4% and mannose 9%. Disregarding arabinose, the composition of hydrolysates from the two modified woods was thus quite similar even though galactose constituted a slightly higher proportion when acetylated wood was the substrate. The composed enzyme mix consequently seems to attack and degrade the substrates in a similar manner.

Fungal decay protection

The indication of a threshold in carbohydrate release around 20-25% WPG due to cell wall bulking by furfurylation corresponds with findings on the decay resistance of furfurylated Scots pine sapwood towards *Coniophora puteana*, see figure 2.

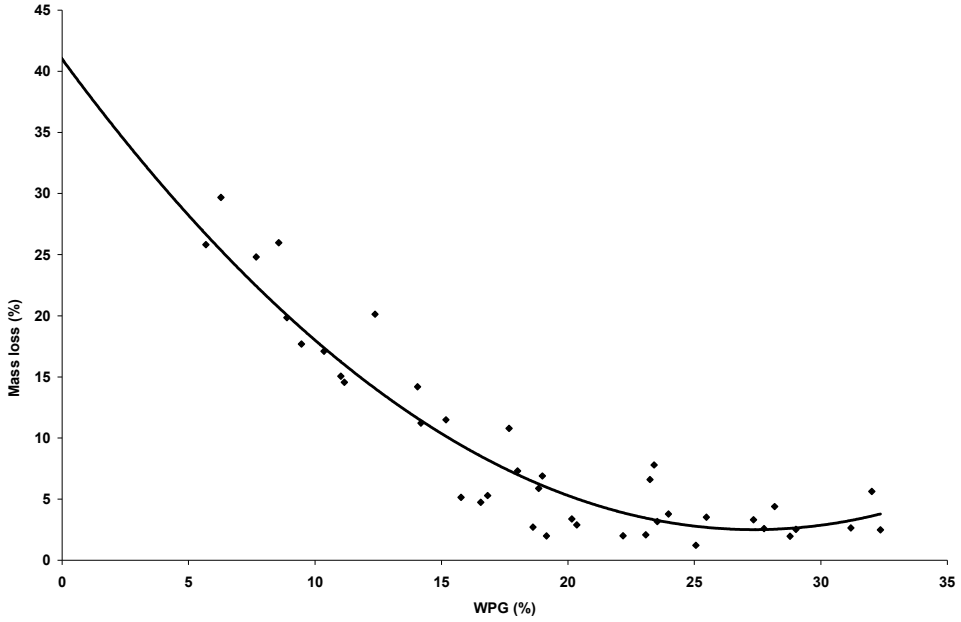

Figure 2: Mass loss percentage (MLP) after 16 weeks of incubation of furfurylated Scots pine sapwood to Coniophora puteana versus weight percent gain (WPG) 2nd order polynial fit, $R^2 = 0.90$.

Due to the above mentioned similarities to the swelling and decay trials, the applied method to assess the enzymatic hydrolyzability may be a viable tool for further investigations regarding the decay protection mechanisms in modified wood. One obvious advantage is that results can be obtained within 48-72 hours after sample preparation. The enzyme mix can be prepared to simulate different decay scenarios and can be further optimized to include other relevant activities for testing of other modified substrates.

CONCLUSIONS

A novel approach to describing mechanisms related to the fungal decay protection offered by wood furfurylation was presented. It was based on the utilization of commercially available isolated enzyme preparations with activities resembling those of

wood destroying fungi. A system was constructed to partly mimic the hydrolytic action of decay fungi. An analytical method was adopted for monitoring the release of monosaccharides due to enzyme catalyzed hydrolysis. The obtained results indicated that moderately furfurylated wood was recognized as a substrate by hydrolytic enzymes with activities resembling those of wood destroying fungi thereby establishing that if adequate moisture is present the substrate will be hydrolyzed. The accessibility of carbohydrates was however reduced markedly which offers a protective effect most likely mainly due to cell wall bulking.

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