Mode of Action of DMDHEU Treatment against Wood Decay by White and Brown Rot Fungi

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ABSTRACT

The resistance of beech and pine wood blocks treated with 1,3-dimethylol-4,5-dihydroxyethylene urea (DMDHEU) against *T. versicolor* and *C. puteana* increased with increasing WPG. Full protection (mass loss below 3%) was reached at WPGs of approximately 15% (beech) and 10% (pine). The infiltration of untreated and DMDHEU-treated wood blocks with nutrients and thiamine prior to fungal incubation did not result in an increased mass loss caused by the fungi. This showed that the destruction or removal of nutrients and vitamins during the modification process has no influence on fungal decay. In order to study the effect of cell wall bulking and increased surface area, the cell wall integrity was partly destroyed by milling and the decay of the fine wood flour was compared to that of wood mini-blocks. The mass losses caused by the fungi, however, also decreased with increasing WPG and showed comparable patterns like in the case of mini-blocks. To study the effect of the chemical change of cell wall polymers, cellulose was treated with DMDHEU and the product was subjected to hydrolysis by a cellulase preparation. The release of sugar during the incubation was clearly reduced as compared to untreated cellulose. Pre-treatment of modified cellulose with Fenton's reagent increased the amount of released sugar due to the cellulase activity. Pine micro-veneers were subjected to Fenton's reagents in acetate buffer over 48 h. While untreated specimens and veneers treated with low DMDHEU concentration displayed strong and steady tensile strength loss, veneers treated to a higher WPG did hardly show tensile strength loss.

INTRODUCTION

High resistance of modified wood against fungal decay is assumed to be due to changes in the material properties of wood rather than a toxic effect on fungal physiology. Three main mechanisms are considered to explain the high decay resistance: (a) reduction of wood moisture content (MC), which impedes fungal colonisation; (b) changes of the cell wall polymers (due to the reaction of hydroxyl groups) that become unrecognisable for enzymes and/or (c) a lower micro-pore size in the wood cell wall that further reduces the accessibility of enzymes as compared to native wood. It is assumed that micro-pore blocking can effectively inhibit the penetration of low molecular weight diffusible agents, which are required for fungal degradation. Incorporation of chemicals into the cell wall leads to cell wall bulking which can be assessed as an increase in the volume

of treated wood (Hill *et al*. 2005). It was the aim of this study to find out, if the prevention of initial fungal colonization, the availability of nutrients, or the structure of the cell wall (surface area, bulking) play the most important role in the anti-fungal efficacy of DMDHEU.

MATERIALS AND METHODS

Wood samples and treatment with DMDHEU

Mini-blocks (30 [long.] x 10 x 5 mm^3) of beech (*Fagus sylvatica* L.) and pine sapwood (*Pinus sylvestris* L.) were treated with aqueous solutions of DMDHEU as previously described (Verma *et al*. 2009). The treatment solutions contained 0.12 mol l^{-1} (7.3 mmol l^{-1}), 0.24 mol l^{-1} (14.7 mmol l^{-1}), 0.36 mol l^{-1} (22.3 mmol l^{-1}), 0.48 mol l^{-1} (30.1 mmol l^{-1}), 0.61 mol l^{-1} (38.0 mmol l^{-1}), 0.83 mol l^{-1} (51.7 mmol l^{-1}) and 1.28 mol l^{-1} (80.3 mmol l^{-1}) of DMDHEU (BASF, Ludwigshafen, Germany) and of $MgCl_2 \cdot 6H_2O$ (concentration given in brackets). After treatment, a leaching procedure was performed according to the EN 84 (1997).

Wood decay test

A mini-block test comparable with the EN 113 (1996) was carried out to assess fungal decay caused by basidiomycetes (Bravery 1978, Verma *et al*. 2008). The wood specimens were incubated with the brown-rot fungus *Coniophora puteana* (Schum.: Fr.) Karst. strain BAM Ebw. 15 (DSM 3085) and the white-rot fungus *Trametes versicolor* (Linneus) Quélet strain CTB 863 A (DSM 3086) at 22°C ± 1°C and 65% RH for 12 weeks. Twelve wood samples for each treatment from four different plates were used to determine the mass loss of the wood blocks.

Effect of nutrients on fungal colonisation

DMDHEU-treated and untreated wood mini-blocks (see above) were soaked with nutrient solution under vacuum (40 mbar, 2 h) and air dried (24 h) as previously described (Verma *et al*. 2008). Two medium solutions were prepared to infiltrate the wood blocks: one solution contained basic nutrients without thiamine, the other basic nutrients with thiamine. The following basic nutrients were dissolved in 1 l of demineralised water: 5.0 g glucose, 2.0 g di-potassium hydrogen phosphate, 0.5 g magnesium sulphate hexahydrate, 0.1 g calcium chloride, 2.7 g sodium acetate, 0.5 g di-ammonium tartrate, 0.025 g magnesium chloride, 0.3 g yeast extract. The nutrient medium was sterilised in an autoclave (120°C, 20 min). The wood blocks without thiamine were sealed in polyethylene bags and sterilized by gamma radiation (25 kGy, Isotron, Netherlands). Aqueous thiamine solution (2 mg ml^{-1}) was sterilised by filtration through a cellulose acetate filter membrane (cut-off 0.2µm, Sartorious, Germany) and added aseptically to the basic nutrient medium.

Decay of wood meal in mesh bags

DMDHEU-treated and untreated wood mini-blocks (see above) were milled to fine powder in a ball mill. The wood powder was put into stainless steel mesh bags (5 x 4 x 0.2 cm^3) with a mesh pore size of 40 µm (F. Carl Schröter, Germany). The bags containing wood powder were dried at 103 °C and the mass was determined. Prior to the fungal test, the bags were sterilized by gamma radiation (25 kGy, Isotron, Netherlands).
The bags were incubated with *C. puteana* or *T. versicolor* in the same manner as the mini-blocks. After 12 weeks incubation, the bags were dried at 103 °C and weighted. Subsequently, the filled bags were again sterilized by gamma radiation, re-inoculated

for another 12 weeks and ML was determined (Verma *et al*. 2008). The nitrogen content of wood powder was determined using an elemental analyser Vario EL III (Elementar Analysensysteme GmbH, Hanau, Germany).

Treatment of cellulose with DMDHEU

Five g of cellulose (Arbocell B 400, J. Rettenmaier & Söhne, Germany) was suspended in 1 l of distilled water and kept overnight to reach maximum swelling. The excess water was drained off and DMDHEU (BASF, Ludwigshafen, Germany) and $MgCl_2 \cdot 6H_2O$ (concentration given in brackets) were added to achieve concentrations of 0.12 g mol^{-1} (7.3 mmol l^{-1}), 0.24 g mol^{-1} (14.7 mmol l^{-1}), 0.36 g mol^{-1} (22.3 mmol l^{-1}), and 0.61 g mol^{-1} (38.0 mmol l^{-1}). The mixture was initially stirred with a glass rod and then overnight with a magnetic stirrer to obtain uniform distribution. The mass was then spread as a thin layer on aluminium foil, pre-dried at room temperature for 72 h and subsequently cured at 120°C for 24 h. The modified cellulose was re-suspended in distilled water and dialysed in dialysis tubes (Medicell 12000-14000 kDa) for 24 h in a 2 l-beaker containing 1500 ml water in order to remove un-reacted chemicals. The water was changed four-six times during the dialysis. After dialysis, the modified cellulose was dried at room temperature (24 h) and finally at 103°C (24 h). The modified cellulose was grinded properly with a mortar and DMDHEU content was determined from the N content. The nitrogen content was determined using an elemental analyzer EA 1108 (Carlo Erba Instruments). The proportion of DMDHEU in treated cellulose was calculated from the total nitrogen content in the sample (Eqn. 1),

$$DMDHEU\,[wt\%] = \frac{N_{cellulose}}{N_{DMDHEU}} \times 100 \qquad (1)$$

where *DMDHEU*: DMDHEU content in modified cellulose; $N_{cellulose}$: nitrogen content in the cellulose (wt %); N_{DMDHEU}: nitrogen content in DMDHEU (15.73 wt%). The nitrogen content of untreated cellulose was below the detection limit (0.05%) of the machine.

Enzymatic hydrolysis of cellulose

Modified and unmodified cellulose (100 mg) were suspended in 50 ml pre-sterilized sodium acetate buffer (100 mM, pH 5.0) in Erlenmeyer flasks (150 ml) and kept overnight, in order to allow maximum swelling. Cellulast 1.5 L (Novozymes, Denmark), a commercial cellulase preparation (56 units), was added to each flask. The flasks were sealed with aluminium foil and kept at room temperature on a rotary shaker at 120 rpm. Liberated reducing sugars were analysed using dinitrosalicylic acid reagent (DNS, Miller *et al*. 1959). Therefore, 2 ml of the suspension was withdrawn at time intervals of 6, 24, 48, 72, 96 and 120h and centrifuged in a glass centrifuge tube (Hettich, Universal 320, Germany) at 4000 rpm for 5 min. One ml of the supernatant and 1 ml of DNS reagent were mixed in a test tube and kept for 10 min in a boiling water bath. Reducing sugar content was analyzed photometrically (Specord 205, Analytic Jena, Germany) at 540 nm and expressed in glucose units. In a second experiment, modified cellulose which had been treated with 0.61 g mol^{-1} DMDHEU solution (DMDHEU content 9.1%) was suspended in 50 ml acidify water (pH 4.0) as described above. Either aqueous H_2O_2 (1% final concentration) or Fenton's reagent (0.5mM of $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$ and 1% H_2O_2, final concentrations) were added and the suspension was kept at room temperature (24h) on a rotary shaker (120 rpm). Subsequently, the suspensions were

filtered through a cellulose acetate filter membrane (cut-off 0.45µm, Sartorious, Germany).
In order to remove excess H_2O_2, the cellulose preparations were washed three time with distilled water, immersed in 50ml distilled water containing 0.5ml of Basopal® 98 (BASF, Germany) for 3h on a rotary shaker and washed again three times with distilled water. The pre-treated cellulose preparations were subsequently treated with 56 unit of the commercial cellulase Cellulast 1.5 L (Novozymes, Denmark) and reducing sugars were determined as described above.

Treatment of micro-veneers with Fenton's reagent

Scots pine micro-veneers were treated with 0.4, 1.2, and 2.0 mol l^{-1} aqueous DMDHEU solutions. Three veneer strips were immersed in 50 ml acetate buffer (0.1M, pH 4.0) in an Erlenmeyer flask (100 ml). Subsequently, $Fe_2(SO_4)_3 \cdot H_2O$ (0.2 mol l^{-1}) and hydrogen peroxide (50 mmol l^{-1}, both final concentration) were added and the flasks were gently shaken in a dark water bath at ca. 25°C. After 0h, 0.5h, 2h, 6h, 24h, 48h, respectively, the treating solution was drained from the flasks, the veneers were washed several times with distilled water and the tensile strength of the wet veneers was determined as described recently (Xie at al. 2007). Six veneer strips were used per treatment and time point. Untreated veneer strips served as controls.

RESULTS AND DISCUSSION

Mass loss of wood blocks

The mass loss (ML) of beech wood caused by *T. versicolor* and *C. puteana* gradually decreased with increasing weight percent gain (WPG), while the untreated control samples displayed a nearly similar ML on all plates (Figure 1a, b). At the highest WPG of approx. 15%, the ML was below 3% for both fungi tested. DMDHEU treatment was somewhat more efficient in pine sapwood (Figure 1c). A WPG of approx. 10% was sufficient to reduce the ML through *C. puteana* below 2%. It was previously shown that reduction in ML was accompanied with a lower degree of fungal colonisation in treated wood (Verma *et al*. 2008).

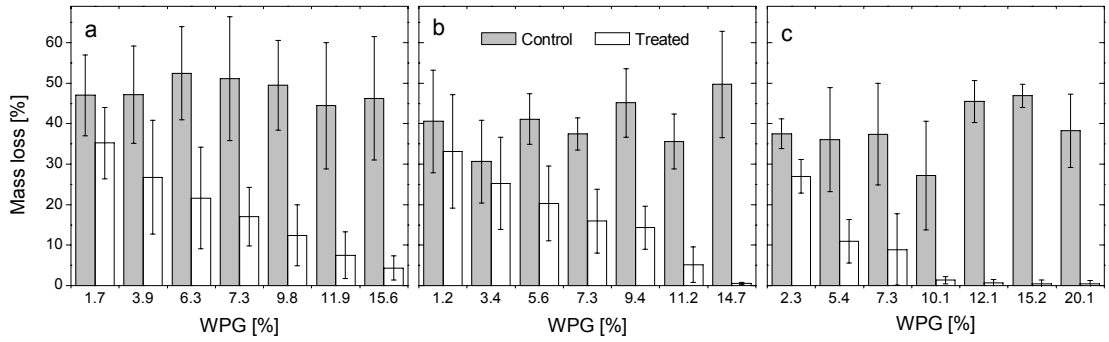

Figure 1: Mass loss of untreated (grey bars) and DMDHEU treated (white bars) mini-blocks after 12 weeks of fungal incubation. (a) T. versicolor, beech; (b) C. puteana, beech; (c) C. puteana, pine (error bars show standard deviation, 12 replicates were used per treatment).

Effect of nutrients on fungal decay

During the first stages of decay, fungi feed on easily available nutrients such as minerals, proteins, simple sugars or vitamins which are deposited in the parenchyma cells. Therefore, untreated and modified wood blocks were soaked with a nutrient

solution and thiamine to support fungal colonisation. The ML of samples with additional nutrients was, however, not significantly higher than in the related blocks without nutrients (Figure 2). Beech samples decayed by *T. versicolor* showed a slight trend of somewhat higher ML with nutrients added (Figure 2a), but this did not occur with pine samples decayed by *C. puteana* (Figure 2b). It might be possible that the nutrients leached out during incubation on the agar plates, but it is unlikely that all added nutrient would leach out, because the blocks were not in direct contact with the medium. The results confirm the hypothesis that high resistance of wood against basidiomycetes is mainly due to change of the cell wall structure, rather than due to a lack of essential nutrients in the beginning of colonisation.

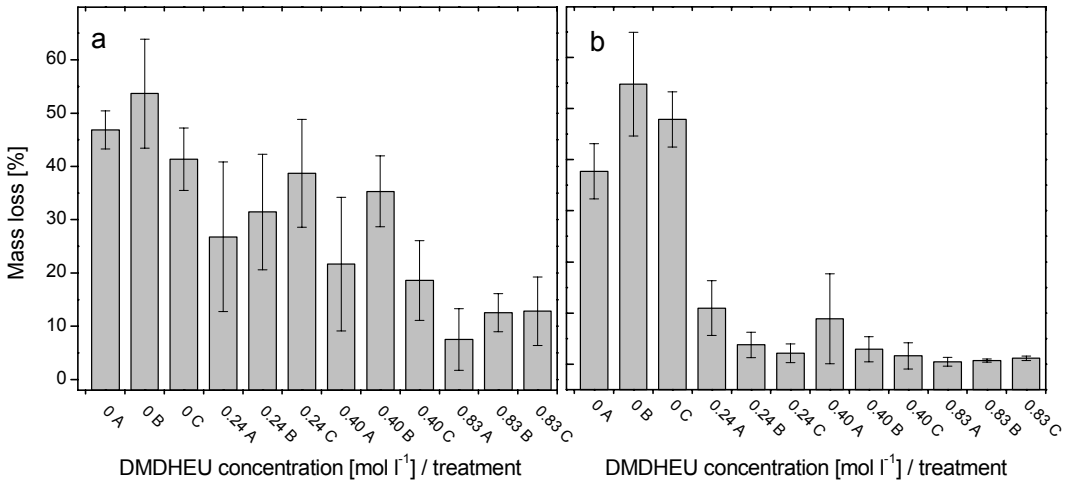

Figure 2: Mass loss of mini-blocks after 12 weeks of fungal incubation (a) T. versicolor, beech; (b) C. puteana, pine. A: without additional nutrients; B: nutrient medium added (without thiamine); C: nutrient medium and thiamine added (error bars show standard deviation, 12 replicates were used per treatment).

Decay of untreated and modified wood flour

Cell wall bulking through incorporated DMDHEU was shown to decrease the size of micro-pores in the cell wall (Dieste *et al*. 2008). It is assumed that micro-pore blocking can effectively inhibit the penetration of diffusible agents with low molecular weight, which are required for fungal degradation (Hill *et al*. 2005). Untreated and modified wood blocks were milled to fine powder in order to increase the surface area and to destroy the cell wall structure. Due to the high surface area of milled wood, extra-cellular enzymes should be able to get in close contact with the cell wall polymers and cause degradation, so that the pore size in the cell wall plays a minor role.

Despite the milling, the ML of the wood meal decreased with increasing WPG of DMDHEU (Table 1). The percentage of ML was somewhat higher than in the respective wood blocks (Figure 1), but also the ML of the meal controls exceeded that of the wood block controls. After 12 weeks incubation, the wood meal samples were incubated for another 12 weeks, in order to examine the long-term effect of fungal colonisation (Table 1). In this second period, the ML of beech (through *T. versicolor*) and pine (through *C. puteana*) modified with 0.24 mol l^{-1} DMDHEU had almost reached the ML of the controls, but the ML of the wood samples with highest WPG was still considerably lower than that of the control. Even at higher WPG (approximately 11%) individual specimens such as *e.g.* beech infected with *C. puteana* reached ML above 50% (Table 1). These ML are clearly higher than those reached with wood blocks

(Figure 1) and might be ascribed to the higher surface area of the wood meal and to the prolonged fungal incubation.

Table 1: Mass loss of untreated (grey bars) and DMDHEU treated (white bars) wood meal in mesh bags after 12 (a, c, e) and 24 weeks (twice 12 weeks; b, d, f) of fungal incubation. (a, b) T. versicolor, beech; (c, d) C. puteana, beech; (e, f) C. puteana, pine (error bars show standard deviation, 9-12 replicates were used per treatment)

WPG [%]	Mass loss [%]					
	T. versicolor, beech		*C. puteana*, beech		*C. puteana*, pine	
	12 weeks	24 weeks	12 weeks	24 weeks	12 weeks	24 weeks
0.0	56.7±14.8	72.7±20.1	48.8±13.4	59.8±12.1	50.9±11.3	59.0±8.6
6.1	39.6±4.6	55.7±6.2	29.3±17.0	39.6±12.4	14.7±6.7	50.8±5.6
11.4	17.9±7.0	26.7±11.8	11.5±4.2	32.8±18.7	10.3±5.7	23.4±10.2
20.3	13.0±4.0	17.6±5.0	8.6±3.0	11.9±6.1	8.4±4.3	13.6±2.0

Enzymatic and chemical degradation of modified cellulose and wood veneers

Untreated and modified cellulose were incubated with a commercial celluolytic enzyme preparation and hydrolysis was determined as amount of released reducing sugars. Untreated cellulose released clearly higher amounts of reducing sugars than modified cellulose (Figure 3a). Although the detected sugars are presented as glucose equivalents, it is assumed that they are mainly released in an oligomeric form. Therefore, the amount of released sugars should be clearly higher that than shown in Figure 3a. The sugar release decreased with increasing DMDHEU content of the cellulose preparation. At low DMDHEU content of 1.9%, the sugar release after 120h was about half of that of unmodified cellulose. The minimum release of sugar was observed at the highest DMDHEU content (9.1%). When modified cellulose (9.1%) was pre-treated with Fenton's reagent, the amount of released sugar was clearly higher compared to the respective not pre-treated preparation.

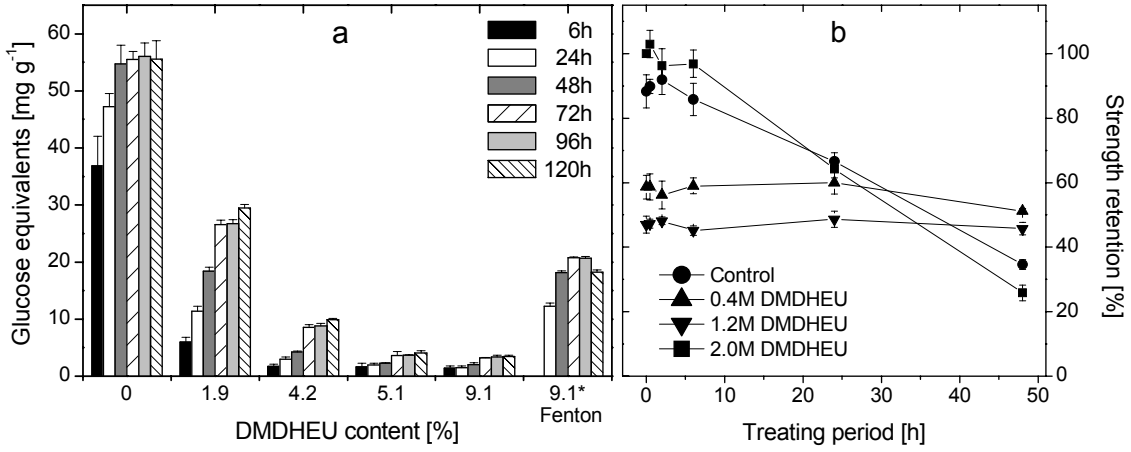

Figure 3: Release of sugar from untreated and DMDHEU-treated cellulose after incubation with a cellulose preparation. The last set of columns (*) was subjected to Fenton's reagent prior to cellulase application (a). Tensile strength loss (tested in a zero-span mode) of pine micro-veneers after incubation with Fenton's reagent (b)

One explanation for the low sugar release from modified cellulose is that the cellulose is chemically changed so that it cannot be transformed by enzymes. Due to the modification, the substrate does not fit into the "pocket" in which the active centre of an enzyme is located. The more bulky the attached chemical is the more difficult is the enzymatic transformation of the altered substrate. DMDHEU-treated and untreated pine

micro-veneers were treated with Fenton`s reagents (hydrogen peroxide and iron salt) which is regarded as an important decay agents involved in brown rot decay (Goodell *et al*. 2008). As reported previously, modification with DMDHEU to higher WPG caused strong tensile strength loss of micro-veneers (Xie *et al*. 2007). However, the controls and the veneers treated to low WPG displayed a severe strength loss in course of the reaction with Fenton's reagent (Figure 3b). In contrast, the specimens treated to a higher WPG showed almost constant tensile strength over the whole treating time. These results show that treatment with higher concentrations of DMDHEU is able to inhibit the degradation of wood by hydroxyl radicals which constitute the most aggressive reduced oxygen species produced in biological systems (Wood 1988).

CONCLUSIONS

It was recently shown that the high resistance of DMDHEU modified wood against decay fungi is accompanied with a low degree of fungal colonisation (Verma *et al*. 2008). The results of these study show that high durability and the minor colonisation cannot be explained by the wash-out or destruction of easy available nutrients during the modification process. A change of the cell wall polymers appears to be the main reason for the high durability.

It was the aim of this study to destroy the cell wall integrity through ball milling, in order to increase the surface area for enzymatic attack. It was assumed that destroying the cell wall and exposing a greater surface area should increase the decay intensity particularly of simultaneous white rot fungi. Still, the mass loss of modified wood with the highest WPG, was not higher after milling compared to that of the wood blocks. These results indicate that bulking of the cell wall and reduction in pore size by DMDHEU is not the main reason for the increased fungal resistance of DMDHEU treated wood. Nevertheless, it cannot be fully ruled out that bulking still plays a role for the decay resistance of modified wood meal. Enzyme accessibility is a molecular scale phenomenon and the size of wood meal particles is still macroscopic.

The modification of cellulose caused a significant reduction of sugar released through the enzymatic action of cellulase. This shows that the anti-fungal resistance of modified wood is not only caused by cell wall bulking, but also by the chemical change of the cell wall polymers itself. The bulky residues attached to cellulose might prevent that the substrate gets into contact with the active centre of the hydrolase. Thus, the bulking phenomenon also occurs on the level of the cell wall polymers. The treatment of modified cellulose with Fenton's reagent increased the subsequent activity of the hydrolase towards the substrate. This can be attributed to a better accessibility of the substrate for the enzyme. Still, the treatment of wood micro-veneers imparted decay protection in terms of attack from hydroxyl radicals produced via the Fenton reaction.

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