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Non-enzymatic depolymerization of cotton cellulose by fungal mimicking metabolites

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ABSTRACT

Small, low molecular weight, non-enzymatic compounds have been linked to the early stages of brown rot decay as the enzymes involved with holocellulose degradation are too large to penetrate the S3 layer of intact wood cells. We investigated the most notable of these compounds, i.e. hydrogen peroxide, iron, and oxalic acid. The former two are involved in the Fenton reaction in which they react to form hydroxyl radicals, which cause an accelerated depolymerization in cotton cellulose. We found the same reaction to be caused by both iron Fe^{3+} and Fe^{2+}. A 10 mM oxalic acid solution showed significant depolymerization effect on cotton cellulose. An oxalic acid/sodium oxalate buffered pH gradient had an inhibitory effect on the reduction of cellulose polymers at increased pH values. The organic iron chelator, EDTA, was found to promote depolymerization of cellulose in combination with Fenton's reagents, but inhibited the effect of oxalic acid in the absence of iron and hydrogen peroxide. Manganese was tested to see if metals other than iron could generate a significant impact on the degree of polymerization (DP) in cotton cellulose. Depolymerizing properties comparable to iron were seen. The results confirm that low molecular weight metabolites are capable of depolymerizing cellulose and suggest an importance of these mechanisms during incipient decay by brown rot fungi.

1. Introduction

Over the past decades, there have been numerous hypotheses to explain the biochemistry and physiology of the processes employed by brown rot fungi during wood degradation. Researchers are getting closer to a unified hypothesis, but the mechanism is yet to be fully understood (Highley and Dashek, 1998; Martinez et al., 2009). Brown rot fungi utilize a symphony of both enzymatic and non-enzymatic components to facilitate wood degradation. With the lack of exocellulytic enzymes, i.e. cellobiohydrolases and β-glucosidases, but in the presence of reactive oxygen species, brown rot fungi cause a rapid depolymerization of cellulose during early stages of wood decay with an accumulation of partially degraded sugars but little initial weight loss (Cowling, 1961; Eriksson et al., 1990).

The initial degradation of cellulose and hemicellulose in the S2 layer of the wood cell wall occurs at a distance from the hyphae. Unfavorably small pore sizes, which limit the ability of brown rot enzymes to diffuse freely through the intact S3 layer, prompted researchers to look for alternative low molecular weight agents capable of penetrating the wood cell wall for initiation of the degradation process (Cowling and Brown, 1969). Low molecular weight, extracellular compounds such as hydrogen peroxide, oxalic acid, biochelators and iron have been demonstrated to play important roles in non-enzymatic degradation (Cohen et al., 2002; Arantes et al., 2010). The Fenton reaction is a key reaction as hydrogen peroxide (H_2O_2) and Fe^{2+} react to generate hydroxyl radicals (Hyde and Wood, 1997; Highley and Dashek, 1998). The hydroxyl radical is the most potent oxidizing agent in most biological systems and capable of increasing the rate of solubilization of cellulose (Wood, 1994; Ritschkoff et al., 1995; Kim et al., 2002) and may be directly involved in the degradation of crystalline cellulose (Itakura et al., 1994; Hirano et al., 2000; Martinez et al., 2009) and other recalcitrant polymers (Kerem et al., 1999).

Oxalic acid ($C_2H_2O_4$) is a low molecular weight organic acid produced as a secondary metabolite in copious amounts by the majority of brown rot fungi (Green and Clausen, 2003; Hastrup et al., 2006). Despite its simple chemical formula the acid has multiple functions. The main roles in relation to the initial

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processes of wood decay are pH regulation, calcium chelation, iron translocation/solubilization, and catalysis of hemicellulose and cellulose hydrolysis (Green et al., 1991; Goodell et al., 1997; Munir et al., 2001). Yet the multiple functions of oxalic acid, as well as its interactions with the compounds in the Fenton reaction, are not fully understood. It has been suggested that high concentrations of oxalic acid might even impede non-enzymatic Fenton-based degradative mechanisms by forming highly stable Fe–oxalate complexes (Arantes et al., 2009).

Fenton chemistry is based on the oxidation of Fe^{2+} to generate hydroxyl radicals. Hence, efficient redox cycling of iron is important in an environment such as wood where Fe^{2+} is a limiting factor. The acidic environment obtained by the production of oxalic acid generates suitable conditions for the Fenton reaction as iron is kept in solution and the autoxidation of Fe^{2+} to Fe^{3+} is avoided (Contreras et al., 2007). Due to the reduced pH during brown rot decay, the metal ions involved are likely to be in a complex such as a siderophore (Highley, 1990). The driving compounds of the Fenton reaction have been characterized as extracellular chelators, which enhance the production of hydroxyl radicals by assisting in the redox cycling of iron (Goodell et al., 2002; Arantes and Milagres, 2006).

The objective of this study is to elucidate the mechanism of direct cellulose depolymerization *in vitro* using chemical mediators produced by brown rot fungi. Non-enzymatic low molecular weight compounds known to be involved in the process were used with cotton cellulose to mimic the action of incipient brown rot decay. The degree of polymerization (number of glucosyl residues per cellulose chain) was analyzed following treatments with one or more of the following compounds: hydrogen peroxide, metal ion (Fe^{2+}, Fe^{3+} and manganese), Ethylene diamine tetraacetate (EDTA) and oxalic acid.

2. Materials and methods

Cotton cellulose samples of 0.2 g were treated in an aqueous solution of different chemicals, which mimic fungal processes during wood decay. Plastic tubes were used to avoid iron extraction from glassware, except in one study where the effect of glass was tested. Deionized, distilled water (ddH_2O) was used for all dilutions and control samples. The samples were incubated in the dark on a rotary shaker at 100 rpm for 24 h at 20 °C unless otherwise stated ($n = 3$). Samples were rinsed with ddH_2O to stop further reaction and to avoid reaction from oxalic acid during overnight drying at 50 °C. The cotton cellulose samples were dissolved in 0.5 M cupriethylenediamine (CED) (GFS Chemicals, Columbus, OH). The viscosity was measured by transferring the sample solution to a 300 Cannon-Fenske viscometer tube (Sigma–Aldrich, St. Louis, MO, USA). The reciprocal of the specific viscosity ($1/n_{sp}$) was calculated by the formula:

$$1/n_{sp} = t_o/(t - t_o),$$

where t and t_o represent the time of outflow of the reaction mixture and buffer, respectively (ASTM, 2003). The specific viscosity was converted to degree of polymerization according to Cowling (1960). All chemicals used were from Sigma–Aldrich (Milwaukee, Wis. USA) unless otherwise stated.

2.1. Time study

The depolymerizing effect of hydrogen peroxide (H_2O_2) on cotton cellulose was tested at concentrations of 0.8 mM, 8 mM or 80 mM for 1, 2, 4, 8, 16 and 24 h. Viscosity was measured as described above.

The influence of iron ions either from glassware or ferric sulfate (Fe^{3+}) was tested. One set of sample was incubated in Pyrex glass tubes with 80 mM H_2O_2. For the remaining tests, the cotton fractions were incubated in plastic tubes with a combination of one or more of the following components: 80 mM H_2O_2, 0.5 mM $Fe_2(SO_4)_3$, 0.5 mM EDTA, 10 mM oxalate. Concentrations of $Fe_2(SO_4)_3$ and EDTA were according to Xu and Goodell (2001). Oxalic acid corresponded to the amounts accumulated by brown rot fungi during wood decay (Hastrup et al., 2006).

2.2. Organic chelator activity and pH study

This series of tests was performed to elucidate the direct effect caused by oxalic acid and the consequence of pH regulation using sodium oxalate buffer. The pH values chosen correspond to the pH value of a gradual shift from sole 10 mM oxalic acid to sole 10 mM sodium oxalate at a ratio of: 1:0 (pH 1.88), 3:1 (pH 2.16), 3:2 (pH 2.45), 1:2 (pH 3.56), 1:3 (pH 4.01), 1:13 (pH 4.67), 1:50 (pH 5.1), 0:1 (pH 7.6). The additive effects caused by the addition of 0.5 mM $Fe_2(SO_4)_3$, 0.5 mM ferrous sulfate (Fe^{2+}), and/or 80 mM H_2O_2 were tested to simulate the Fenton reaction. Iron and H_2O_2 concentrations were chosen according to Xu and Goodell (2001).

The influence of pH/oxalic acid on iron chelators was performed with 0.5 mM EDTA either alone or in combination with H_2O_2 and Fe^{2+} or H_2O_2 and Fe^{3+}.

Acetate buffer (10 mM) was used as alternative buffer to view the direct effect of this buffer compared to the sodium oxalate buffer (pH 4.2).

2.3. Manganese study

Manganese (Mn) was tested as an agent alternative to iron in the Fenton reaction. Cotton cellulose was treated with 0.5 mM manganese (II) sulfate (Mn^{2+}) (99% purity) at pH values described above.

2.4. X-ray diffraction

For XRD measurements, the treated, dried cotton was pressed into a cylindrical wafer 25 mm in diameter and 4.3 mm thick. The cotton wafers were scanned according to Howell et al. (2009). The spectra were processed and analyzed using a Rietveld analyzing method (Rietveld, 1967, 1969) using the cellulose 1β crystal structure published by Nishiyama et al. (2002). Percent crystallinity was calculated from XRD spectra by comparing the area of the crystalline regions to the total area.

2.5. Statistical analysis

All statistical analyses were performed using Student's two-tailed t test. P-values ≤ 0.05 were considered significant. Results are presented as the mean value of the replicates ± standard error or error bars representing the standard error.

3. Results and discussion

3.1. Time study

Cotton cellulose controls were tested at zero time (2181 ± 117 DP) and at time points as presented in Fig. 1 (dH_2O-treated samples) and the results are in agreement with previous study (Green, 2000). We found hydrogen peroxide (H_2O_2) capable of depolymerizing cotton without the addition of iron, which may be due to the presence of indigenous metals, including iron, in cotton cellulose (Brushwood and Perkins, 1994). Increasing the

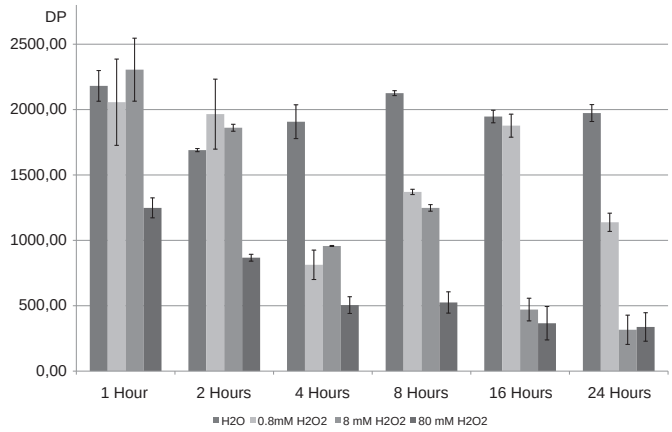

Fig. 1. Time study on the depolymerizing effect caused by H_2O_2 on of cotton cellulose at concentrations: 0 mM, 0.8 mM, 8 mM, or 80 mM ($n = 3$).

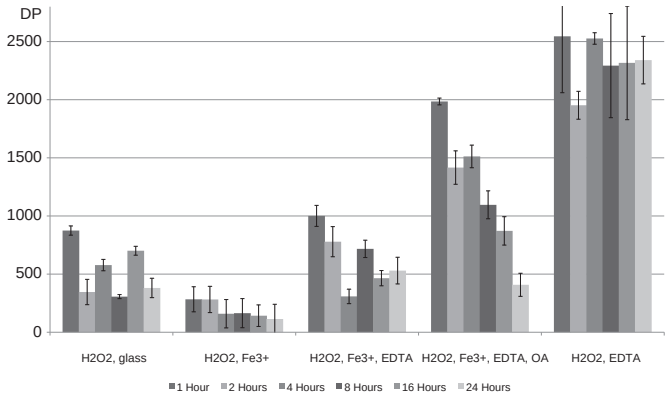

Fig. 2. Treatment of cotton with H_2O_2 (80 mM) and combinations of glass, $Fe(SO_4)_3$, EDTA, and OA over a period of 1–24 h of exposure. The degree of polymerization of untreated cotton cellulose control samples at time zero were 2181 ± 117 DP ($n = 3$). The average DP of dH_2O-treated control measured at time 1–24 h was 1970 ± 71 DP ($n = 18$).

concentration of hydrogen peroxide resulted in an increased or accelerated depolymerization of the cotton cellulose (Fig. 1). The highest H_2O_2 concentration (80 mM) resulted in rapid depolymerization of the cotton cellulose with a significant reduction observed after 1 h of incubation. Solutions with 8 mM and 0.8 mM H_2O_2 showed continuous reduction of DP throughout the incubation period. After 16 h, no statistically significant difference was observed between cotton treated with 8 mM and 80 mM (p-value > 0.1). The exponentially decreasing effect observed for H_2O_2 is not solely a result of a reduction in the cleaving potential but also due to the mode of action, i.e. random cleavages in the cellulose chain. In early stages of depolymerization, cleavages at points remote from the chain ends will cause relatively large decrease in viscosity compared to cleavages later when the average cellulose chain is shorter. Thus, cellulose chain depolymerization measured as reduced viscosity will occur at an increasingly slower rate even if the number of cleavages remains constant (Arantes and Milagres, 2006).

In our study the effect of hydrogen peroxide alone does not diminish instantaneously, but a continuous though exponentially decreasing depolymerization of the cotton cellulose was observed over the period of 24 h most notable for cotton treated with 8 mM H_2O_2. Also, a continued reduction in polymerization was observed in Fenton-mediated reactions (Fig. 1). This indicates ongoing though declining formation of reactive oxygen species throughout the incubation period. Qian et al. (2004) measured the free radical activity over time using ESR spin-trapping techniques and reported a rapid reduction in the formation. In the chelator (2,3-dihydroxybenzoic acid) mediated system, they found the hydroxyl radical formation to last longer with continued detectable free hydroxyl radical generation. The prolonged effect of the hydrogen peroxide in our study may be a consequence of the presence of natural iron reductant in cotton cellulose.

Addition of both iron and the use of glass tubes for incubation accelerated the initial depolymerizing effect of H_2O_2 significantly (p-value < 0.05) (Fig. 2), however the enhanced turnover by these factors was diminished after 4 h of incubation. The reaction from glass could be due to leaching of borosilicate glass composites e.g. Fe, silicon (Si) and aluminum (Al). These may act as a catalyst for hydrogen peroxide development. The amplified depolymerization of cotton as a result of iron treatments or metal leaching from glassware supports the involvement of Fenton chemistry in the degradation of cellulose (Koenigs, 1972).

The combination of H_2O_2 with EDTA resulted in a notable though overall insignificant (p-value > 0.05) increase in DP (viscosity) in comparison to the control samples (Fig. 2). The difference between H_2O_2 and H_2O_2/EDTA-treated samples was highly significant (p-value < 0.001). This indicates that EDTA is preventing or slowing the amount of depolymerization possible by chelating the indigenous metals in the cotton cellulose e.g. Fe or calcium making them unavailable for the Fenton reaction. This EDTA complex could cause an increase of the viscosity and result in an apparently higher DP. EDTA has previously been reported to have a negative effect on fungal decay (Viikari and Ritschkoff, 1992).

Despite an initially slow depolymerization of cotton, the presence of iron-chelating compounds (EDTA and OA) resulted in an ongoing depolymerization with a continuous decrease from 1 to 24 h (p-value < 0.05). The effect was most pronounced in treatments with a combination of EDTA and oxalic acid. The binding of metal ions, in this case Fe^{3+}, resulted in a slow initiation of depolymerization, as little free iron was available for Fenton chemistry. Yet a significant depolymerization was seen over the 24 h period (p-value < 0.02). Formation of calcium oxalate crystals may be another explanation for the initial slowing of the process (Green and Highley, 1997) as these may cause an overall increase of viscosity of the cotton. However, the results imply that these solutions maintain the capacity to break up cellulose bonds possible due to ongoing •OH production. This can be due to continuous reduction of ferric iron to ferrous iron throughout the test period as a result of the presence of chelators (Xu and Goodell, 2001; Contreras et al., 2007). Though it should be noted that EDTA itself is non-reducing and oxalic acid induced iron reduction is a light dependent reaction (Hyde and Wood, 1997). In relation to wood degradation the presence of chelators would allow for a more efficient reaction as •OH radicals are produced continuously at a slow rate. The likelihood that these react with substrate is therefore higher than with a system comprised solely of H_2O_2 and Fe^{3+} where •OH are produced quickly and mainly react with themselves by radical formation (Contreras et al., 2007).

3.2. pH study

A 10 mM oxalate solution (pH 1.88), simulating the pH value generated in wood after just one week of decay by brown rot fungi (Green et al., 1991; Hyde and Wood, 1997), shows a significantly higher degradative effect on cotton than solutions buffered with sodium oxalate at higher pH (p-value < 0.05) (Fig. 3). Oxalic acid (pH 1.88) has a seemingly direct impact on the β-1,4-cellulose bonds. This depolymerizing effect of oxalic acid is supported by

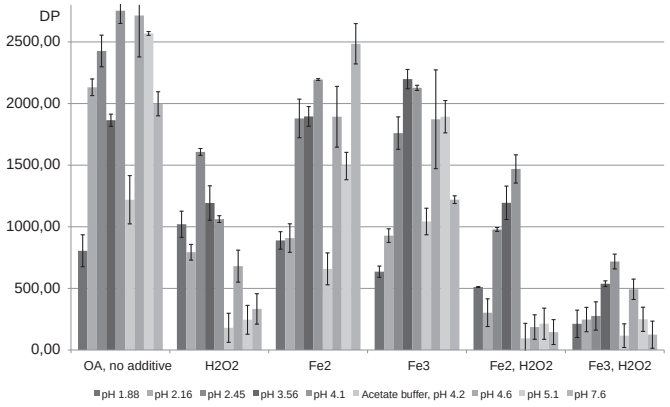

Fig. 3. Treatments with oxalic acid (10 mM) with either no additive or in combinations with H_2O_2, Fe^{2+}, or Fe^{3+}. Treatment solutions with pH values other than 1.88 are in oxalate/sodium oxalate buffered or acetate buffered media.

other reports showing similar reduction in DP of cellulose (Shimada et al., 1991; Green et al., 1992, 1993; Jordan et al., 1996). The direct destructive effect of oxalic acid on the cotton cellulose may be due to its role as a proton source for non-enzymatic hydrolysis of carbohydrates and as a metal chelator (Shimada et al., 1997). The inhibiting effect of oxalic acid at high concentrations or high oxalate/Fe ratios found by other researches (Shimada et al., 1997; Xu and Goodell, 2001) was not pronounced in this study. When cotton balls are oven-dried, the heat concentrates the oxalic acid, which then increases the depolymerizing effect as the balls dry out (Green et al., 1992). To avoid this, the chemically exposed cotton cellulose in this study was rinsed in distilled water before drying overnight to remove excess oxalic acid.

The combination of H_2O_2 with either Fe^{2+} or Fe^{3+} in the OA-buffered solution gave a significant reduction in DP. The effect of H_2O_2 is most pronounced at pH values of 4.2 and higher. The absence of an added effect of H_2O_2 in combination with pure oxalic acid (pH 1.88) compared to Fig. 1 may be linked to the chelating effect of OA making the iron naturally present in the cotton unavailable for the Fenton reaction and the formation of calcium oxalate (Green et al., 1991). The effect seen for treatments with Fe^{2+} without the presence of H_2O_2 may be due to autoxidation as Fe^{2+} oxalate in aerobic solution that lead to hydroxyl radical formation. Fe^{2+} reacts with O_2 by one-electron transfer forming superoxide ($O_2^{\bullet -}$), which is rapidly removed by reaction with Fe^{2+} thus creating H_2O_2. Hence, partial autoxidation creates the Fe^{2+}/H_2O_2 combination required for hydroxyl radical production (Hyde and Wood, 1997). The autoxidation of Fe^{2+} is pH and chelator dependent and in the very acidic environment close to the hyphae, Fe^{2+} is reported to be resistant to autoxidation with a lifetime of an hour or more (Hyde and Wood, 1997). In terms of Fe^{3+}, it will form a complex with oxalic acid at acidic pH that in the presence of light can lead to oxidation of oxalate and reduction of Fe^{3+} (Schmidt et al., 1981; Wood, 1994). The samples in this test were incubated in dark conditions but not shielded entirely from light during mixing and takeout. Perhaps this brief exposure to light caused slight photochemical damage to the cotton cellulose.

The assays in this part of the work were carried out at pH between 1.88 and 7.6 to resemble the pH range found in wood during decay (pH 1.88–5.1, approximately) (Hyde and Wood, 1997) and beyond (pH 7.6). As wood decay fungi secrete oxalic acid, the pH close to the fungal hyphae is reduced dramatically (~pH 2) with a gradual increase toward the pH of wood (approximately pH 5.0–5.5). Non-enzymatic biodegradation has been suggested to take place around pH 5.0 (Goodell et al., 1997; Hyde and Wood, 1997; Xu and Goodell, 2001). High generation of hydroxyl radicals is unlikely in the acidic pH in close proximity to the fungal hyphae as the sequestration of iron by oxalate may limit the production of hydroxyl radicals in this region (Qian et al., 2002). The Fenton reaction is thereby inhibited, preventing an otherwise harmful reaction between •OH and the fungal hyphae (Fig. 3, Fe^{2+}, H_2O_2 at pH 1.88).

Oxalate concentration and pH are known to affect the chelation and reactivity of Fe^{3+} (Hyde and Wood, 1997). Though at very acidic pH the efficiency of treatments with Fe^{3+} and H_2O_2 were less affected due to both solubilization of bound Fe^{3+} by oxalic acid (Arantes and Milagres, 2006) and the formation of a complex with two oxalate ions. This Fe^{3+}–oxalate complex has a redox potential that allows reduction to Fe^{2+} by fungal chelators (Hyde and Wood, 1997). Above pH 3 Fe^{3+} forms stable complexes with three oxalate ions that are almost certainly not reducible by fungal chelators, thus disrupting the non-enzymatic Fenton-based degradative mechanism (Varela and Tien, 2003).

3.3. Organic chelator activity

EDTA is used in this study to simulate the effect of an organic, fungal produced chelator, which forms a stable complex with metals, i.e. iron or Mn, and accelerates the iron-dependent formation of hydroxyl radicals by lowering the redox potential of Fe^{3+}/Fe^{2+} (Sutton and Winterbourn, 1984). The chelator concentration was kept equivalent to the concentration of iron. In the fungal environment the chelator:iron ratio may be carefully controlled both by controlling oxalic acid production for the solubilization and sequestration of iron and the production of low molecular weight iron reducing phenolate chelators (Connolly et al., 1996; Qian et al., 2002). The presence of EDTA causes a significant increase in depolymerization in complex with Fe^{2+}/H_2O_2 and Fe^{3+}/H_2O_2 at pH values between 2.45 and 4.1 (Fig. 4) in comparison with the same treatments without EDTA (p-value < 0.05) (Fig. 3). This could be due to the reduction of Fe^{3+} by oxalic acid (Schmidt et al., 1981; Goodell et al., 1997; Hyde and Wood, 1997). In a similar experiment, higher yields of hydroxyl radicals were found in the presence of EDTA comparable to experiments without EDTA, which may explain the decrease in DP (Wood, 1994; Li et al., 2007). In most biological systems, iron is generally sequestered in redox-inactive complexes to prevent oxidative damage via Fenton chemistry. However, this is not the case in fungal degraded cellulose or wood likely due to the pH gradient. The process is induced by available

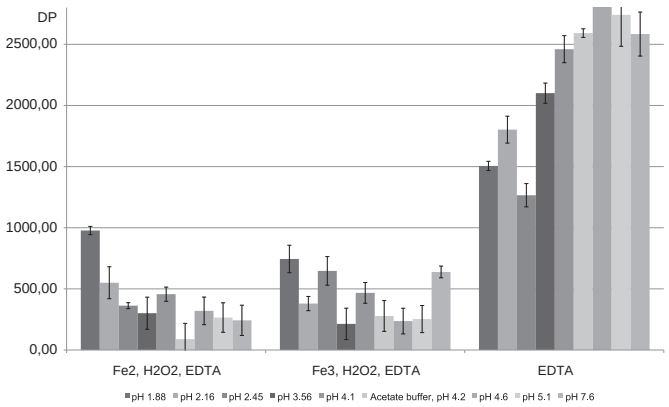

Fig. 4. Treatments with combinations of H_2O_2 (80 mM), Fe^{2+}(0.5 mM), Fe^{3+}(0.5 mM), and EDTA (0.5 mM) in sodium oxalate or acetate buffered media. Controls are the same as in Fig. 3 (non-additive treatment).

chelators and reductants, which sequester and keep iron in solution and make HO• radical generation feasible (Hammel et al., 2002).

The ferric-EDTA complex exposed to H_2O_2 performed comparatively to the ferrous–EDTA complex; we did not find the significantly lower rate reported by Gutteridge (1985). He found the EDTA complex formed by pre-mixing a ferrous salt with EDTA to endorse deoxyribose degradation compared to a complex prepared with EDTA and a ferric salt. In the absence of hydrogen peroxide, they found the iron complex prepared from the ferrous salt to autoxidize to the ferric state and thereby inhibit deoxyribose degradation (Gutteridge, 1985). However, in our case the components were added simultaneously which prevented the Fe^{2+}–EDTA complex from autoxidizing prior to H_2O_2 addition. This could explain the differences observed.

The effect of EDTA alone (Fig. 4), compared with the results from treatments with no additives in oxalic/sodium oxalate buffer (Fig. 3), showed a reduction in DP for pH values of 2.16 and 2.45. This supports the arguments for autoxidation at the low pH values presented above. So even though EDTA is considered to be an inhibiter of iron-dependent reactions, it has been found to form a concentration dependent complex with iron that promotes oxidative reactions and promotes autoxidation of iron (Goodell et al., 1997).

In a control study, we used acetate buffer as a reference since it has previously been used for studies of cellulose degradation by Fenton chemistry (Koenigs, 1974; Wood, 1994; Xu and Goodell, 2001). For most treatments, acetate buffer (pH 4.2) significantly lowered the DP in comparison with the same treatment performed in sodium oxalate buffer at a pH of the same order of magnitude (pH 4.1 and 4.6). Only treatments with EDTA alone showed no differences between the acetate-buffered and sodium oxalate-buffered samples. Apparently, the presence of EDTA resulted in a significant inhibition of the compound or compounds in acetate buffer responsible for the observed reduction in polymerization of cotton. Acetate can work as a chelator though weaker than most organic acids (Wood, 1994). Displacement by stronger chelators, i.e. EDTA, can lead to lower redox potentials and induced autoxidation. May be the enhanced performance observed for treatments with acetate buffer compared to oxalate/sodium oxalate and the EDTA treated samples is due to a weak chelation making iron more readily available for Fenton chemistry. Acetate buffer has previously been used as a solvent for analysis of the chemical effect on cellulose. Xu and Goodell (2001), who found the same effect in acetate buffer (10 mM) at pH 4.2, explained the enhanced reduction in DP as due to the strong binding of cellulose with Fe^{3+} and little binding with Fe^{2+}.

3.4. Manganese as an alternative to iron

In wood decay, iron is the most commonly associated catalyst in the application of Fenton's reagent but other metals may also play a role. Modified Fenton's reactions (80 mM H_2O_2 and 5 mM $MnSO_4$) were conducted at a range of pH regimes using oxalic acid/sodium oxalate buffer to investigate the potential of soluble manganese (Mn^{2+}) as a catalyst for the generation of hydroxyl radicals. Incubating cotton cellulose in Mn-solution causes a bi-phasic decrease in the degree of polymerization of the cellulose, as is the case with iron. The effect is even further enhanced when hydrogen peroxide is added to the solution (Fig. 5). The same depolymerizing effect was seen for other metals (zinc and aluminum) in combination with H_2O_2 (data not shown). This confirms the observation that other transition metals than iron are capable of catalyzing the decomposition of hydrogen peroxide (Watts et al., 2005). However, Mn^{2+} was not found to react with H_2O_2 to form •OH (Gutteridge and Bannister, 1986), though our data indicate that it may be an alternative for the growth of fungi in Fe-deprived environments. Manganese is a transition metal that exists naturally in the +2 and +4 oxidation states. Similar to iron, manganese is found in the soil. Watts et al. (2005) found soluble manganese to promote hydroxyl radical production at acidic pH regimes; though, significantly higher concentrations of catalyst are required compared to iron-catalyzed reactions.

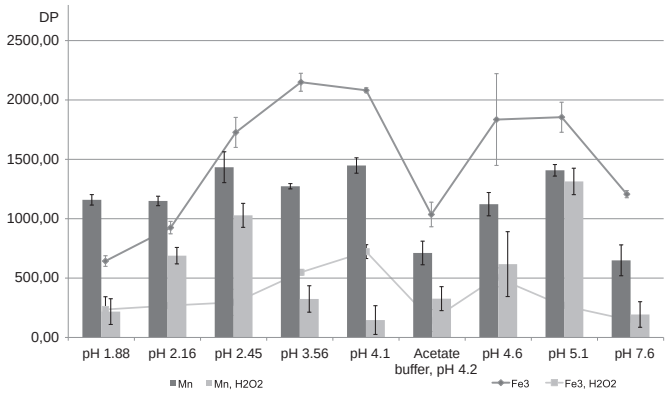

Fig. 5. Manganese treatment of cotton with and without H_2O_2 in a sodium oxalate buffer or acetate buffered (pH 4.2) ($n = 3$). The controls are the non-additive treatment in Fig. 3.

3.5. Structural change in cellulose crystallinity

Analyses done by X-ray diffraction were unsuccessful in detecting a reduction in percent crystallinity relative to the reduction in polymerization ($p > 0.06$). Even samples with an 80% reduction in the degree of polymerization displayed only small changes in crystallinity (Table 1). Similar tests by Green et al. (1992) also failed to detect a decrease in crystallinity index commensurate with decreased DP of native cellulose treated with cell-free culture filtrate from *Postia placenta*, Fenton's reagents or organic acids. Moreover, tests performed by Murmanis et al. (1988) found wood cell walls to be visually degraded by H_2O_2–Fe^{2+} but showing no measurable weight loss. Highley (1977) found no sugar release during cellulose depolymerization. The results could be the result of two different mechanism taking place in the cellulose as a response to the treatment: One indicating that Fenton-produced reactive molecules, such as hydroxyl radicals, can cause a break in the major cellulose chain-bond linkages. Such breakages have a major impact on the DP but do not affect the crystallinity, causing significant sugar release or result in measurable weight loss. This

Table 1
X-ray diffraction analysis of chemically mediated cotton cellulose. Samples were fractions of samples in sodium oxalate buffer, pH 2.56–4.1 presented in Figs. 3 and 4. The percent crystallinity is a mean of the results obtained from samples incubated in buffers at pH 2.45, 3.56, and 4.1. Standard error is presented as ±.

	% Crystallinity
Control samples, untreated	75.01 ± 1.01
H_2O_2	73.57 ± 0.85
Oxalic acid buffer	72.54 ± 1.07
Fe_{2+}	72.48 ± 0.74
Fe_{3+}	73.14 ± 1.03
H_2O_2, Fe_{2+}	74.26 ± 0.36
H_2O_2, Fe_{3+}	73.76 ± 0.55
H_2O_2, Fe_{2+}, EDTA	70.67 ± 4.95
H_2O_2, Fe_{3+}, EDTA	73.32 ± 1.19
EDTA	70.69 ± 2.69

has led to the assumption that brown rot fungi rely on 1,4-glucanases to solubilize cellobiose to glucose (Mansfield et al., 1998; Valásková and Baldrian, 2006). Another contributor to the seemingly unchanged crystallinity could be a consequence of the preferential removal of the easily available outer portions of the microfibrils with a non- or paracrystalline structure (Larsson et al., 1997). Removal of this material will cause an initial increase in crystallinity (Howell et al., 2009) and make the overall measurable changes in crystallinity index appear less.

4. Conclusion

Cellulose depolymerization by non-enzymatic fungal compounds is a complex process with many facets and aspects. This work focused on some of these and presented the result of the influence and interacting effect of H_2O_2, iron, organic chelators (oxalic acid and EDTA) and pH on the depolymerization of cotton cellulose.

This study shows that even at low concentrations H_2O_2 causes depolymerization of cotton over a period of 24 h. The effect of H_2O_2 is enhanced by the addition of Fe^{2+} or Fe^{3+}. The results give a strong indication that the Fenton reaction is a key element in cellulose depolymerization during brown rot decay. Some differences are observed in the depolymerization capacity of Fe^{3+} and Fe^{2+}, with Fe^{+3} being more potent than Fe^{+2} at lower pH values in sodium oxalate buffed solutions. This is argued to be due to the reducing/chelating capacity of oxalic acid (Koenigs, 1972, 1974; Schmidt et al., 1981). Oxalic acid had a direct impact on degree of polymerization of cotton, however in the combination with Fenton chemicals ($Fe^{2+/3+} + H_2O_2$) at pH values between 2.45 and 4.1 less depolymerization was observed relative to higher pH values. This range corresponds to the pH generally generated by brown rot fungi in the proximity of the hyphae and the inhibition of Fenton chemistry may be seen as a defense mechanism against otherwise harmful reactive oxygen species. Even though hydroxyl radical production occurs in both very acidic ($pH < 4$) and mildly acidic ($pH > 4$) environments, oxalic acid sequestration of iron in the lower pH environment around the fungal hyphae may limit the production of •OH in this region (Goodell et al., 1997).

Autoxidation, oxalate complex formation and varying chelator availability are speculated to play an important role in the depolymerization observed at low pH values (Wood, 1994), which could cause a problem to biological systems. Our results with the iron chelators (EDTA) resulted in reduced DP at low pH values (2.16–3.45) suggested to be due to autoxidation of iron. Fungal chelators have been reported to cause an increase in the reaction rate for the reduction of iron lower pH (Goodell et al., 1997). However, *in vivo* a pH-buffered environment helps prevent the formation otherwise harmful hydroxyl radical in close proximity to the hyphae and the hyphal sheath surrounding the fungal hyphae serve as additional protection (Green et al., 1993).

The influence of OA/pH on the Fenton reaction, solubility of iron, and metal chelators in fungi has been studied for decades but the mechanisms involved still need to be fully elucidated, as the difference in OA secretion and pH reduction in the adjacent environment can be highly variable among the different species of wood decaying brown rot fungi. Further research into this field is needed to determine whether these differences can be correlated with the type and amount of chelators that are produced.

The crystallinity did not reflect the measured degree of polymerization and theories on removal of non- and paracrystalline material along with location of the breakage points may explain this, however little is still known about how and where in the crystalline cellulose structure non-enzymatic compounds attack. The direct impact on the crystallinity index and the cellulose structure by these agents is an area of great impact for further utilization of cellulose compounds.

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