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Section 1 **Biology**

Chemical mediated depolymerization of cotton cellulose for the understanding of non-enzymatic fungal decay

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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 found to be too large to penetrate the S3 layer of intact wood cells. The most pronounced of these which were analyzed in this study are hydrogen peroxide, iron, and oxalic. The compounds related to the Fenton reaction: the combination of hydrogen peroxide and iron caused marked lowering of the degree of polymerization in the cotton cellulose after treatment. This was the case for both iron ions; Fe^{3+} and Fe^{2+}. A 10mM solution of oxalic acid also showed significant depolymerizing effect on cotton cellulose, whereas diluting the oxalic acid with sodium oxalate to obtain a pH gradient, showed that this decreased the effect reducing of oxalic acid. In addition an organic iron chelator, EDTA, was tested but was found to inhibit depolymerization when in solution with chemicals related to Fenton chemistry. Manganese was tested to see if other metals than iron could generate an significant impact on the degree of polymerization of cotton cellulose and the metal showed good depolymerizing properties as a substitute for iron. We conclude that low molecular weight metabolites are capable of effectively depolymerizing cellulose during incipient decay by brown-rot fungi.

Keywords: hydrogen peroxide, oxalic acid, depolymerization, Fenton reaction, cotton cellulose

1. INTRODUCTION

Brown-rot decay is characterized by rapid and extensive depolymerization of hemicellulose and cellulose in the initial stages of wood decay. Although brown-rot fungi lack exo-1,4-glucanase, which is specific for hydrolyzing crystalline cellulose or natural cellulose, they still preferentially degrade cellulose in wood causing rapid strength loss (Cowling 1961). However, there are numerous hypotheses to explain the biochemistry and physiology of the degradative systems employed by brown-rot fungi for cellulose degradation and the mechanisms are still being investigated (Highley and Illman 1991, Highley and Dashek 1998, Martinez *et al*., 2009). Though the pore size in wood is too small for known brown-rot enzymes to diffuse freely through intact wood cell walls (Cowling and Brown 1969, Flourney *et al*. 1991), the polysaccharides in the S2 layer of the wood cell wall are initially degraded at a distance from the hyphae of brown-rot fungi without any visible decay of the S3 layer. This suggests that brown-

rot fungi produce low molecular weight, extracellular agent(s) capable of diffusing through the wood cell wall and initiating the degradation process. Labeled antibody, Immuno-TEM, demonstrated that the extracellular cellulase from brown-rot fungi was limited in wood cell walls except in advanced stages of decay (Kim *et al*. 1991, Srebotnik and Messner 1991). Hence, it has been suggested that brown-rot fungi produce extracellular degradative agents of low molecular weight. These are capable of crossing the S3 layer from the cell lumen and diffusing into the S2 layer, and might be involved in the loosening of the cell wall structure in the initial stages of fungal attack (Green and Highley 1997, Hirano *et al*. 2000). Iron, hydrogen peroxide, biochelators and oxalate are believed to play important roles in cellulose degradation by brown-rot fungi (Xu and Goodell 2001).

Koenings (1972) reported that hydrogen peroxide (H_2O_2) was a likely factor involved in brown rot decay and linked it to Fenton chemistry. In the reaction, H_2O_2 plays an important role in the generation of hydroxyl radicals in reaction with a metal, traditionally Fe^{2+} but others may be just as potent (Hyde and Wood 1997, Highley and Dashek 1998). It is suggested that hydroxyl radicals might be involved in the degradation of crystalline cellulose (Koenigs 1974, Itakura *et al*. 1994, Hirano *et al*. 2000, Martinez *et al*. 2009) and recalcitrant polymers (Kerem *et al*. 1999) by brown-rot fungi. Recent studies indicate that extracellular H_2O_2 is produced by brown rot fungi and diffuse into the wood cell walls in the early stages of decay and in the combination with Fe^{2+} cause an initial cellulose depolymerization (Ritschkoff *et al.* 1995, Kim *et al*. 2002).

Oxalic acid ($C_2H_2O_4$) is a small, organic acid which is produced in copious amounts by the majority of brown-rot fungi as a secondary metabolite (Takao 1965, Bech-Andersen 1987, Green and Clausen 2003, Hastrup *et al*. 2006). Oxalic acid (OA) and hydrogen peroxide were reported to be formed simultaneously and highest amounts of oxalic acid (10mM and hydrogen peroxide (39.5 μM) were obtained on amorphous cellulose after 3 weeks cultivation (Ritschkoff *et al.* 1995).
Oxalic acid has, despite its simple chemical formula, multiple functions. Three main roles that have been proposed are the chelation of calcium from pectin, reduction of iron from Fe^{3+} to Fe^{2+} and lowering of the pH in wood to around 1.6-2.5 for acid catalysed hydrolysis of hemicellulose and cellulose (Green *et al*. 1991, Munir *et a*l. 2001). Green *et al.* (1991) showed that the pH of wood could be lowered to 1.7 in as little as one week after colonization by *Postia placenta* (Fr.) M.J. Larsen and Lombard (1986), strain MAD-698. This pH approaches the lower pKa of oxalic acid, e.g. 1.23. Oxalic acid has been shown to chelate both calcium and copper (Green and Clausen 2003, Clausen and Green 2003). In addition, it has also been suggested as a translocator of Fe^{3+} (Goodell *et al*. 1997, Arantes *et al*. 2009) and an electron donor. All these aspects also makes oxalic acid an important candidate as a low molecular weight metabolite in brown rot decay. However, the multiple functions of oxalic acid as well as the interactions with the compounds in the Fenton reaction are not fully understood.

The objective of this study is to elucidate the aspect of cellulose depolymerization by biomimicking the mechanisms of low molecular weight compounds produced during incipient brown-rot decay. This will be done by investigating the effect on cotton cellulose of hydrogen peroxide in combination with iron (Fe^{2+} and Fe^{3+}), EDTA and oxalic acid through a period of 24 hours. Other metals such as manganese may work as a substitute for iron in Fenton reaction. In addition, the influence of oxalic acid in brown rot decay is well supported, however other acids are present and their ability to influence the degree of polymerization (DP) of cotton will be viewed in comparison to oxalic acid.

2. MATERIALS AND METHODS

Cotton cellulose samples of 0.2 g were treated with a solution of different chemical biomimicking fungal processes during wood decay. The total volume of the solution was 10 ml (n = 3). The samples were incubated in a 30-ml test tube for 24 hours unless otherwise stated.Plastic tubes were used to avoid iron extraction from glassware except in one study where the effect of glass was tested. Water treatment was used as control. The tubes were placed on a rotary shaker at 100 rpm. After incubation the samples were rinsed twice with dH_2O to stop further process and avoid any reaction from oxalic acid during drying overnight at 50°C. A 0.05-g fraction of the cotton cellulose was placed in plastic tubes. 5 ml dH_2O was added prior to 5 ml 1M Cupriethylenediamine (CED), a cotton cellulose solvent. The cotton was dissolved after 20 minutes at 25°C with occasional shaking. The sample was kept at 25°C during testing. A #300 Cannon-Fenske viscometer tube (Sigma-Aldrich, St. Louis, MO, USA) was used to measure the viscosity of the liquid (ASTM OD 1795-96, ASTM 2001). The degree of polymerization of the cotton cellulose was calculated according to Cowling (1960).

2.1 Time study

The influence of varying hydrogen peroxide (H_2O_2) concentrations over time was tested. 0.2g cotton cellulose fractions were treated with H_2O_2 at concentrations of 0.8mM, 8mM and 80mM, and incubated at room temperature for 1, 2, 4, 8, 16 and 24 hours, respectively. Viscosity was measured as described above.

Further tests were done with a variety of low molecular weight compounds normally present in the natural wood or produced by the fungi during wood decay. These compounds have been associated with cellulose degradation (Highley 1977, Ritschkoff *et al*. 1995, Xu and Goodell, 2001). Iron either from glassware or as $FeSO_4$ (Fe^{3+}) was tested as the principal component as it is directly related to H_2O_2 in the Fenton reaction. In one setup, cotton cellulose fractions was incubated in glass tubes with 80mM H_2O_2. In the remaining tests the cotton fractions were incubated in plastic tubes with a combination of one or more of the following components; 80mM H_2O_2, 0.5mM $FeSO_4$, 0.5mM Ethylene diamine tetraacetate (EDTA), 10mM oxalate. Oxalate and EDTA were used to represent organic chelators. EDTA, at concentrations as described by Xu and Goodell (2001). Oxalic acid (OA) was used at concentrations similar to what is accumulated by brown rot fungi during wood decay (Hastrup *et al*. 2006). Control samples were incubated and measured as described above.

2.2 pH study

This test was performed to extrapolate on the direct effect caused by oxalic acid (OA) and the consequence of regulating the pH at different levels using sodium oxalate as buffer. The pH values chosen corresponded to the pH value of a gradual shift from sole 10mM oxalic acid to sole 10mM sodium oxalate at a ratio of: pH 1.88 (100:0), pH 2.16 (3:1), pH 2.45 (3:2), pH 3.56 (1:2), pH 4.01 (1:3), pH 4.67 (1:13), pH 5.1 (1:50), pH 7.6 (0:100). The direct effect of OA at different pH values was found using dH_2O. The additive effects caused by an addition of 0.5mM ferrous sulphate (Fe^{2+}), 0.5mM ferric sulphate (Fe^{3+}), with and without 80 mM H_2O_2 were tested to simulate the Fenton Reaction. Iron and H_2O_2 concentrations were according to Xu and Goodell (2001).
10mM acetate buffer was used as alternative buffer to view the direct effect of this buffer compared to the sodium oxalate buffer at approximately the same pH (pH 4.2).
The cotton was incubated for 24 hours at room temperature. Depolymerization (DP) measured as described by Cowling (1960).

Magnesium was tested as analternative to iron in the Fenton reaction. Cotton cellulose was treated with 0.5mM $MnSO_4$ at the same pH values as described above.

Additional tests were run to test the effects of EDTA as a representative of an organic iron chelator, and was used at the concentration (0.5mM) used by Xu and Goodell (2001).

3. RESULTS

3.1 Time study

The highest H_2O_2 concentration (80mM) gave a rapid reduction in the degree of polymerization (Fig. 1), whereas 0.8mM and 8mM H_2O_2 showed an initial increase in DP indicating polymerization of cotton. After 24 hours of incubation the 0.8mM solution of H_2O_2 had halved the degree of polymerization, which was the same effect observed for 80mM H_2O_2 after just 2 hours. There was no statistically significant difference between 8mM and 80mM after 24h, indicating a maximum reduction of the cellulose polymers to limit of DP. Based on this it was decided to incubate the cotton for 24 in the further tests.

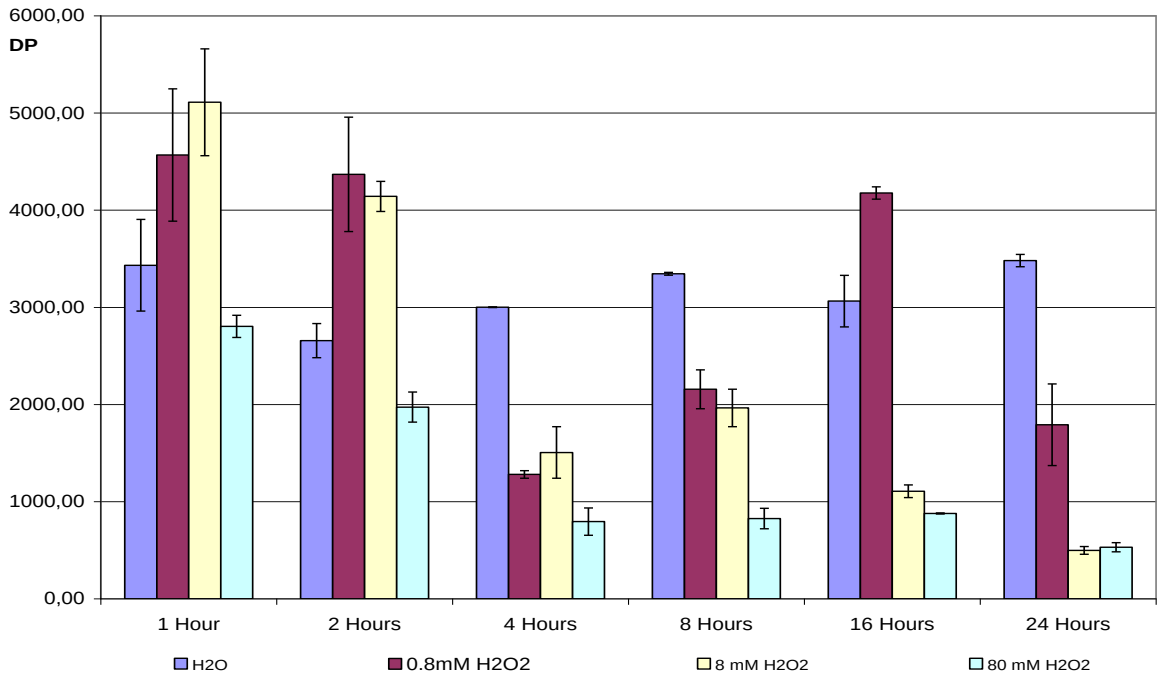

Figure 1: Time study of the influence of different concentrations of H_2O_2: 0mM, 0.8mM, 8mM, or 80 mM incubated for 1, 2, 4, 8, 16 or 24 hours on the depolymerization of cotton cellulose.

Additional tests in the time study with $FeSO_4$ or the use of glass tubes during inoculation showed a positive effect on the break up of cotton cellulose polymers (Fig. 2).The initial faster depolymerization of cotton treated in the glass tubes compared to plastic tubes also with H_2O_2 was leveled out after 4 hours of incubation. Cotton treated with a combination of $FeSO_4$ and 80mM H_2O_2 showed a significant reduction in the degree of polymerization relative to cotton treated just with a 80mM H_2O_2 solution. Noticeably, the initial depolymerization is amplified by the addition of iron. These results support the involvement of the Fenton reaction in the degradation of cellulose. The addition of both EDTA and OA resulted in an initial increase in DP indication a binding of EDTA to the iron present in the cellulose making it unavailable for Fenton reaction and reducing the viscosity of the solution; However, by 24 hours some

depolymerization had occurred. In the sample with no added Fe^{3+} no such decrease is observed. Also the oxalic acid may bind the iron or react with the calcium resulting in the formation of calcium oxalate crystals. The calcium oxalate crystals will be dispersed in the sample and may influence the measurement, resulting in a higher viscosity e.g. higher calculated DP.

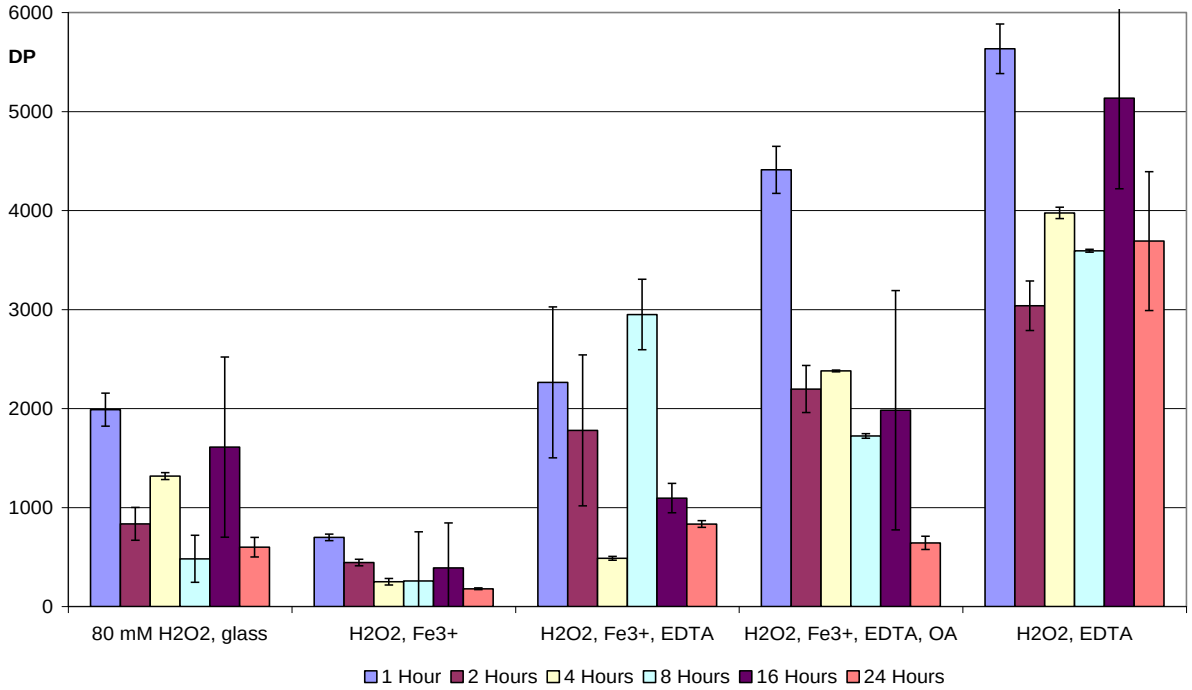

Figure 2: Treatment of cotton with a combination of H_2O_2, iron, EDTA, and OA over a time period of 1, 2, 4, 8, 16, or 24 hours.

3.2 pH study

In treatments solely with the oxalate buffer solution (Fig. 3, H_2O) oxalic acid does not cause any markedly changes in DP except in the pure 10mM solution, pH 1.88. Seemingly, oxalic acid can destroy the β-1,4,cellulose bonds.

As in the time study, H_2O_2 was capable of reducing the DP both alone and in combination iron but only at suitable pH values. The effect of H_2O_2 and the generated free hydroxyl radicals is most pronounced at pH values of 4.2 and higher. At pH 1.88 H_2O_2 does not seem to have any effect without iron present. The acidic solutions at pH 1.88 - 4.1 reduce the depolymerizing power of the Fenton reaction. This was most pronounced in reactions with Fe^{2+}+ H_2O_2 but was also seen to some extend in the solution with Fe^{3+} and H_2O_2 at pH 3.56 - 4.6.
The tests showed that treatments with Fe^{3+} and H_2O_2 are more effective in depolymerizing the cotton cellulose than Fe^{2+} and H_2O_2 at low pH thus diminishing the reducing role of oxalic acid. Iron treatments (Fe^{3+} and Fe^{2+}) with no H_2O_2 present showed only small effects on the DP except for pH 2.16, which was significantly different from the control. Adding H_2O_2 clearly increased the effect. The combination of iron and hydrogen peroxide worked better than either component alone.

Acetate buffer enhanced the effect of all the treatments compared to treatments with oxalate buffer at similar pH values (4.2 for acetate buffer vs. 4.1 and 4.6 for oxalate buffer).

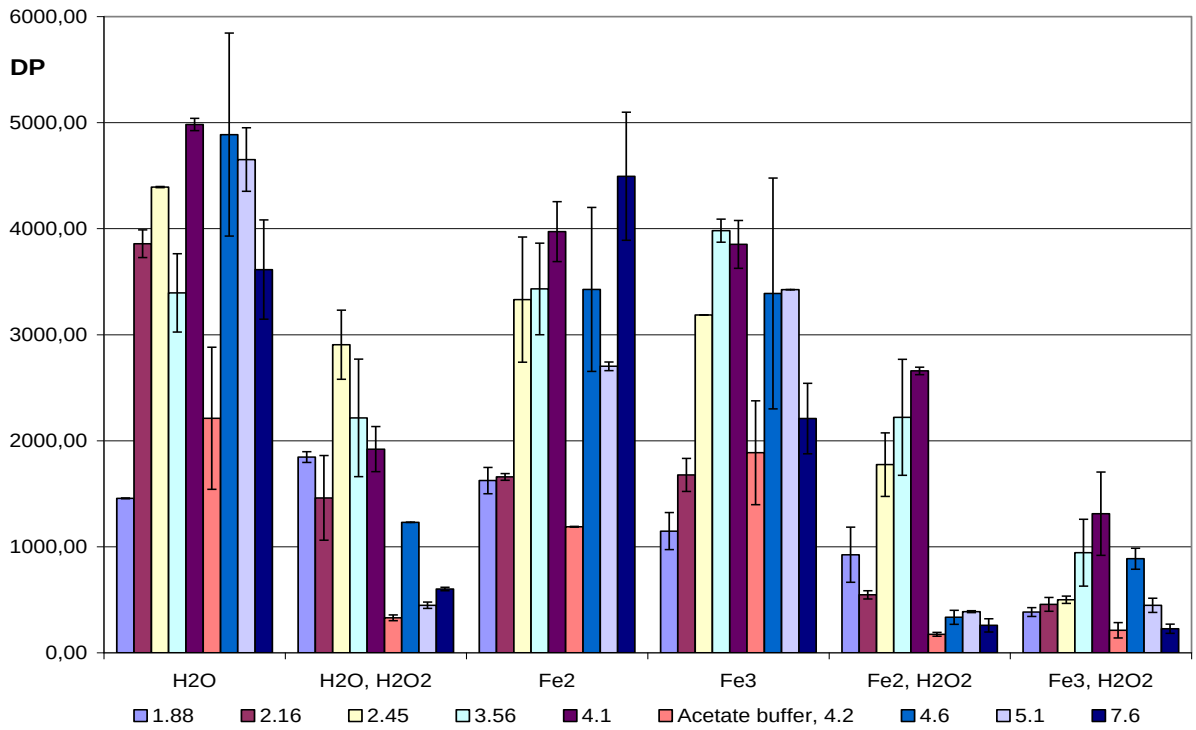

Figure 3: Treatments with a combination of H_2O_2, Fe^{2+}, Fe^{3+} in oxalate/sodium oxalate or acetate buffered media.

3.4 EDTA study

Ethylene diamine tetraacetate is used in this study to simulate the effect of an organic, fungal produced chelator, which captures and facilitates the transport of metals, i.e. iron/Mn. The depolymerization of cotton by Fe^{2+} and H_2O_2 is increased when EDTA is present in the solution with (Fig. 4). This can be a result of the chelation of the iron making it more available for the H_2O_2 in the Fenton reaction. In solutions with Fe^{3+} and H_2O_2 EDTA does not make much difference. Cotton treated solely with EDTA in the pH varying buffer solutions presented no difference compared to the H_2O treatment in Fig. 3.

3.3 Manganese as an alternative to iron

Incubating cotton cellulose in a manganese solution causes a bi-phasic decrease in the degree of polymerization of the cellulose structure as is the case with iron and the effect is even further enhanced when hydrogen peroxide is added to the solution (Fig. 5). The same depolymerizing effect were seen for other metals in combination with H_2O_2 (data not shown).

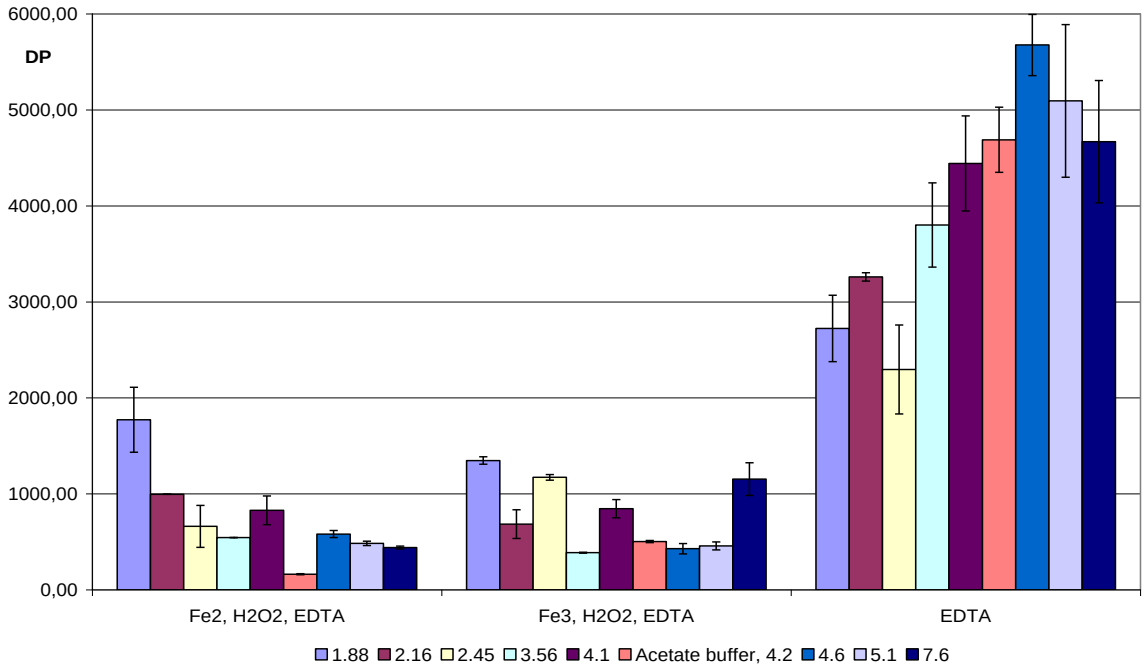

Figure 4: Treatments with a combination of H_2O_2, Fe^{2+}, Fe^{3+}, and EDTA in sodium oxalate or acetate buffered media.

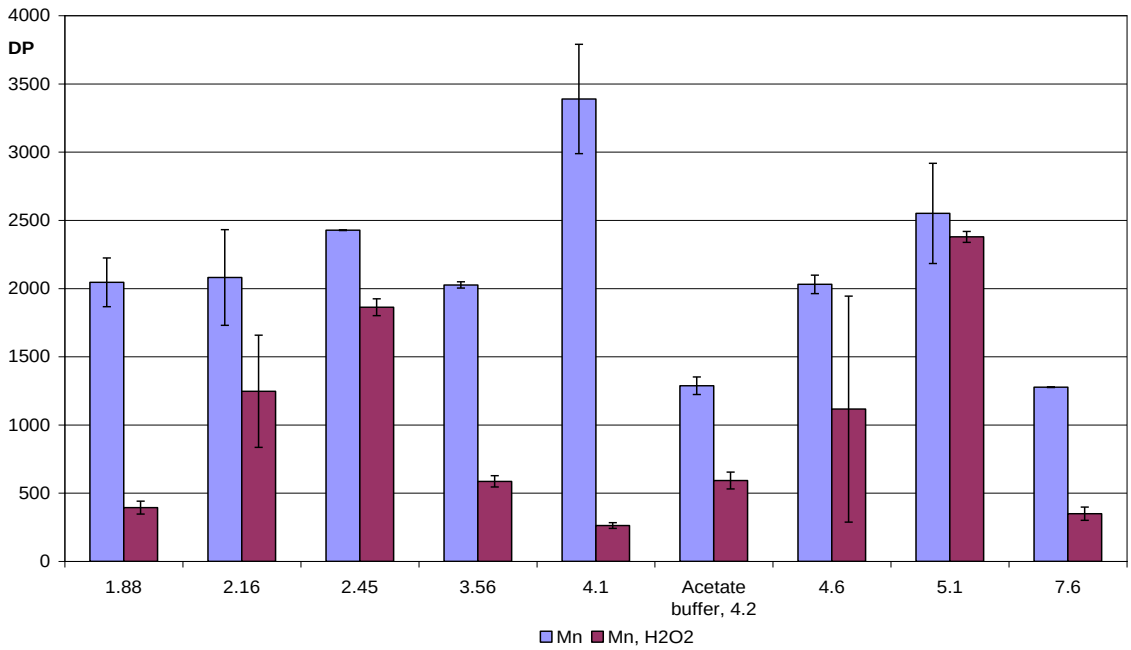

Figure 5: Manganese treatment of cotton with and without H_2O_2 present in an oxalate buffer solution at different pH values (n = 3).

4. DISCUSSION

The setup with H_2O_2 and iron mimicking the Fenton reaction definitively shows significant depolymerization, the same was seen when using other metals such as Manganese. However tests performed by the authors using X-ray diffraction did not find any changes in the percent cellulose crystallinity or in the spacing between the crystalline planes (data not shown). Green *et al*. (1992) also failed to see a decreased crystallinity index commensurate with decreased DP of native cellulose treated with *P. placenta*, Fenton reagents or acids. Tests performed by Murmanis *et al*. (1988) found wood cell walls to be degraded by H_2O_2-Fe^{++} but weight loss was not measurable. Highley (1977) likewise presented depolymerized cellulose but no sugars was released in the process indicating, that only major chain-bond linkages are broken by the hydroxyl radicals supposably generated in the reaction. This leads to the assumption that presumably glucanase enzymes for breaking up the cellubiose into glucose.

The pH value found to have the most destructive effect (pH 1.88) simulates the pH value monitored in wood after just one week of decay generated by brown rot fungi. The pH here is lowered to around 1.6-2.5 mainly as a result of the accumulated oxalic acid (Green *et al*.1991, Hyde and Wood 1997). This significant depolymerizing effect of oxalic acid is supported by other rapports showing similar reduction in DP of cellulose (Shimada *et al.* 1991, Green *et al*. 1992, 1993, Jordan *et al*. 1996) and hemicellulose (Bech-Andersen 1987). The results indicate that theoretically, the higher the OA concentration the better in terms of direct effect. Again, this would say that the more OA is produced by fungi the faster the decay, but calcium precipitation may be required to protect the hyphae (Bech-Anderson 1987). This may be the case for some fungal species, however is does not apply for *Gloeophyllum trabeum* (Pers.) Murrill (1908). *G. trabeum* has a low accumulation of oxalic acid during decay and the pH in wood under attack stays around 4.0 (Clausen and Green 2003). The direct destructive effect of oxalic acid on the cotton cellulose may be due to the hydrolyzing effect of the acid. It is found that when cotton balls are oven-dried, the heat concentrates the oxalic acid, which then increases the depolymerizing effect as the balls dried out (Green *et al*. 1992). To avoid this, the chemically exposed cotton cellulose in this study were rinsed in distilled water before drying over night. Thus, whereas most brown-rot fungi make excess OA, *G. trabeum* may produce excess H_2O_2 for DP.

The reduced depolymerizing effect of Fe^{2+} and H_2O_2 at low pH values is interesting, as this is the range to which most brown rot fungi lower the pH in the wood during wood decay. However, it corresponds to the hypothesis by Hyde and Wood (1997) with the lowest pH closest to the fungal hyphae and then a gradual increase toward the pH of wood (approximately pH 5.0-5.5) where the low pH values prevent the fungal hyphae in having direct contact with otherwise harmful hydroxyl radicals. Goodell (2003) stated the hypothesis that an abrupt pH differential between the lumen and the wood cell wall may apply with a pH gradient being formed in the wood cell wall.

The results seen in the cotton cellulose treated with a combination of H_2O_2 and oxalic acid (Fig. 3), may be linked to the chelating effect of the acids or the binding of calcium, as the degree of polymerization was unaffected compared to the control. The iron present in the cotton which would otherwise react with the H_2O_2 is tied up and unavailable for the Fenton reaction.

Acetate buffer has previously been used as a neutral solvent (Xu and Goodell 2001). However, results from this study shows that acetate buffer markedly enhanced the effect of all the

treatments comparatively to treatments with oxalate buffer at similar pH values. This makes it unsuitable in this study as a neutral solvent for control. The reason for this enhancing role may be the reduced chelation binding of iron by acetic acid compared to oxalic acid which makes the chemicals; iron and H_2O_2, more available to Fenton chemistry.

5. CONCLUSION

This study clearly shows that even low concentrations of H_2O_2 can lower the degree of polymerization of cotton over 24 hours**.** The effect of H_2O_2 is further enhanced when adding Fe^{++} or Fe^{3+} as reagent. As many others before us, we also believe that the Fenton reaction is a key element in cellulose depolymerization during brown rot decay. Fe^{3+} is found more effective compared to Fe^{2+} in several of the tests. It is interesting to see that Fe^{+3} was more potent than Fe^{+2} at lower pH values i.e. in solutions with more oxalic acid present to work as a reducing agent (Koenig 1972, 1974). The results indicate that Fenton chemistry works even at low pH values of 1.88 in the presence of oxalic acid although at a slightly reduced rate.
Manganese was found to be an excellent substitute for iron in combination with H_2O_2.
We found acetate buffer at pH 4.2 with H_2O_2 in solution to effectively depolymerize cotton and this may help explain how low oxalic acid producers like *G. trabeum* can effectively degrade wood possible by producing excess H_2O_2 for depolymerization.

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