Iron sequestration in brown-rot fungi by oxalate and the production of reactive oxygen species (ROS)

Yuan Zhu^{1,2}, Liangpeng Zhuang², Barry Goodell^{2,*}, Jinzhen Cao^{1,*}, James Mahaney³

¹MOE Key Laboratory of Wooden Material Science and Application, Beijing Forestry University, Qinghua East Road 35, Haidian 100083, Beijing, China

²Dept. Sustainable Biomaterials, Virginia Tech, Blacksburg, VA. USA

³Edward Via Virginia College of Osteopathic Medicine, Blacksburg, VA. USA

*Corresponding Author

Abstract:

The biochemical role of oxalate in the fungal solubilization of iron is well known, but additional information is needed on the role that oxalate plays in the brown-rot decay of lignocellulose. In this study iron sequestration from iron and iron oxide-hydroxides was assessed with regard to the function of a chelator-mediated Fenton (CMF) reaction and the generation of reactive oxygen species (ROS). The reactions conducted at the lowest pH condition (pH 2.0) and oxalate concentration (15μM) generated the greatest amount of FeII, suggesting that pH, rather than oxalate concentration may have a greater impact on Fenton reactions, and specifically the CMF reaction. Moreover, iron oxide-hydroxides that were pre-incubated with oxalate prior to the CMF reaction, generated more ROS than the iron oxide-hydroxides without oxalate incubation. This indicates that an enhanced CMF reaction was promoted by iron sequestration due to oxalate incubation, and also supports previous research showing a pH-dependent iron transfer from oxalate to chelators produced by fungi. Although Fenton reactions were enhanced at low pH, the iron transfer reaction was enhanced at higher pH. In a decay environment, this would limit damaging Fenton reactions from occurring in the low pH environment directly surrounding the fungal hyphae.

Key words:

Oxalate; Iron oxide-hydroxides; Iron sequestration; Brown-rot fungi; Reactive oxygen species

1. Introduction

The mechanism of lignocellulose biodegradation is of great interest because of the need to find new environmentally sound methods for the protection of wood and other lignocellulose materials (Goodell et al., 1997; Schultz et al., 2007), and because of interest in bioconversion of lignocellulose in biorefinery applications (Goodell et al., 1997; Ray et al., 2010; Sánchez, 2009). One of the most destructive types of decay, brown-rot decay is characterized by a rapid decrease in strength in early stages because of the extensive holocellulose depolymerization produced within the wood cell wall (Arantes et al., 2012; Cowling, 1961; Zhou et al., 2011). However, all degrading enzymes, including those of brown-rot and white-rot fungi, are too large to penetrate the unaltered microstructure of the cellulosic cell wall, preventing enzymatic attack in early stages of wood degradation (Blanchette, 1995; Highley and Dashek, 1998). Non-specific oxidizing chemistries are known to depolymerize cell wall components; however these chemistries would also be destructive to the fungal hyphae. Research has indicated however

that brown-rot fungi typically generate their oxidizing chemistries at a distance from the fungal mycelia (Hammel et al., 2002; Hyde and Wood, 1997; Kim et al., 1991). (Arantes et al., 2009)

Several studies have demonstrated that Fenton-like reactions involving H_2O_2 and FeII produces reactive oxygen species (ROS), which are the primary oxidizing agents involved in cell wall attack (Hammel et al., 2002; Irbe et al., 2011; Jensen et al., 2001; Koenigs, 1974). Further, research shows that low molecular weight degrading agents produced by brown-rot fungi are capable of diffusing into the wood cell wall to participate in fungal degradation (Goodell et al., 1997; Goodell et al., 2003; Ritschkoff, 1996). This evidence has led to the development of research on a non-enzymatic extracellular mechanism for initial brown-rot decay, known as the chelator-mediated Fenton (CMF) reaction (Arantes et al., 2010; Goodell et al., 1997; Jensen et al., 2001; Xu and Goodell, 2001).

For the CMF to be active in generating ROS though, iron must be available to the organism to be reduced by reductants. Although iron oxide-hydroxides are abundant in most soils and wood, these types of iron are unavailable to most microorganisms until solubilized. In response to iron deficiency, many microorganisms have developed specific iron acquisition systems to sequester iron from its oxide-hydroxide forms by secreting iron high-affinity chelators. Some organisms produce compounds known as siderophores for this purpose (Boukhalfa and Crumbliss, 2002; Crowley et al., 1991; Guerinot and Yi, 1994; Hider, 1984), but other non-siderophore chelators can be generated by fungi as well. Sequestration of iron is important for metabolic processes, and in the brown rot fungi, it is also essential for the CMF reaction to be effective (Arantes et al., 2010; Goodell et al., 1997; Goodell et al., 2003).

Shimazono et al. (1952) first observed the accumulation of oxalate secreted by wood-destroying fungi, which later was identified in the cultures of many different brown-rot and white-rot fungi (Connolly and Jellison, 1995; Espejo and Agosin, 1991; Takao, 1965). Oxalate is not produced by all fungi but has been shown to be involved with fungal decay systems linking degradation mechanisms with multiple ecological functions (Dutton and Evans, 1996; Shimada et al., 1994). Although other roles for oxalate undoubtedly exist, the CMF system supports a role for oxalate in sequestering iron from insoluble oxidized forms to form Fe-Oxalate complexes (Goodell, 2014; Goodell et al., 1997; Hyde and Wood, 1997). Under appropriate conditions, mediated by a pH shift which occurs between the fungal extracellular matrix (ECM) region and the wood cell wall, iron will be transferred to catecholate/hydroxyquinone chelators to be reduced and initiate the Fenton reaction (Goodell et al., 1997; Hyde and Wood, 1997). The CMF system in the brown rot fungi therefore requires activity from two different types of chelators (Goodell, 2014). Additional information is needed, however, on the role that oxalate plays in the iron transfer reactions, and how this is affected by both oxalate concentration and pH.

Limited research on the iron sequestration by oxalate produced by brown-rot fungi has been conducted. Besides promoting the iron sequestration from its oxide-hydroxides, simultaneously, oxalate can also suppresses the reduction of sequestered iron by chelators dependent upon concentration and pH (Arantes et al., 2009; Varela and Tien, 2003). It is therefore important to better understand iron sequestration by oxalate and the subsequent reduction of iron by catecholate/hydroxyquinone chelators in the presence of oxalate.

The primary objective of this research was to better understand how iron is sequestered from iron oxide-hydroxides by oxalate over time, and how catecholate chelators may then

access this iron.

2. Material and methods

All reactions in section 2.2-2.4 were conducted in the dark as oxalate would photo-reduce iron in bright light. 2,3 dihydroxybenzoic acid (2,3-DHBA) was used as previously described to mimic the catecholate chelators produced by brown rot fungi.

2.1 Chemical reagents

2,3-DHBA, and ferrozine were purchased from Acros Organics (NJ, USA). 5,5-dimethyl-1-pyrroline N-oxide (DMPO) was purchased from Sigma Aldrich. Acetic acid and hydrochloric acid (HCl) were purchased from Spectrum Chemical Manufacturing Corp. For other chemical reagents, including oxalate, potassium chloride (KCl), sodium acetate trihydrate, ferric chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), hydrogen peroxide (H_2O_2), were all purchased from Fisher Scientific, Fair Lawn, NJ. To obtain naturally occurring iron oxide-hydroxides, freshly prepared FeCl_3 [designated as Fe(Fre)] was exposed to oxygen for at least one month to produce oxygenated iron compounds, [designated as Fe(Oxy)].

2.2 Iron transfer from oxalate to 2,3-DHBA

Fe(Fre)-oxalate was produced by incubating Fe(Fre) with oxalate at a molar ratio of 1:1.5 for 24h. This ratio was selected to insure chelation of the iron by oxalate. A kinetic study of iron transfer and reduction was then conducted by mixing the iron oxalate with 2,3-DHBA in buffer solution. Two buffers were used: pH 2.2 - Clark and Lubs buffer (100mM); and pH 4.4 – acetate buffer (100mM). Ferrozine was used as a specific detection agent for FeII. Buffered ferrozine was added last to the mixture, and FeII generation over a 24h period was determined against standards at 562 nm (NanoDrop 2000c). The final reaction mixture contained 30 μM FeCl_3 , 45 μM oxalate (except for the control), 2 μM 2,3-DHBA and 250 μM ferrozine reagent.

2.3 ROS production with 2,3-DHBA and oxalate-sequestered iron and detection by spin-trap EPR spectroscopy

The ROS spin-trap, DMPO (100 mM final concentration) was mixed with Fe(Oxy)-oxalate and 2,3-DHBA (30 μM). The two buffers and iron solution were made as overviewed in section 2, with Fe(Oxy)-oxalate produced using 30 μM FeCl_3 and 45 μM oxalate. H_2O_2 (1.5mM) was then added to the reaction mixtures to initiate ROS generation. ROS generation was determined using the spin-trap DMPO and the characteristic 2-line signal for the DMPO spin adduct was detected using electron paramagnetic resonance (EPR) spectroscopy. EPR spectra of the trapped radicals were recorded at room temperature after 5 min of reaction time using a Bruker Biospin E-scan X-band EPR spectrometer. The following settings were used, center field 3468; sweep width, 100G; microwave attenuation, 10dB; modulation frequency, 86 kHz with 1G modulation amplitude; time constant 5.12 msec and a receiver gain of 4480. All spectra were averaged over 16 scans for each sample. Spectra were digitized and recorded to PC using the E-scan software WinEPR, and were analyzed using the same program.

2.4 Iron sequestration by oxalate

Fe(Oxy) was mixed with oxalate in an expanded buffer range of 2.0, 3.5, 4.8, and added to appropriately buffered 2,3-DHBA (as listed in Table 1), with ferrozine added last to monitor FeII generation over a 24 hour span as described in section 2.2. Because ferrozine permanently binds FeII, but not FeIII, as iron is reduced it shifts the reaction conditions by removing FeII from the reaction. For these experiments, this shift was desirable as a means of mimicking the reaction of FeII with oxidants like H₂O₂, which would be present in actual brown-rot decay environments, and also would repeatedly react with any FeII generated, oxidizing it to FeIII.

Table 1. Samples for iron sequestration by a kinetic Ferrozine assay. Three 100mM buffer solutions were used: pH 2.0 (Clark and Lubs buffer) and 3.5 and 4.8 (both acetate buffers) and all samples were made up to 3 ml.

3. Results and discussion

3.1 Transfer of iron from oxalate to 2,3-DHBA

Freshly prepared FeCl₃ was mixed with 2,3 DHBA either with or without oxalate. FeCl₃ (without oxalate) in the presence of 2,3-DHBA at pH 2.2 was reduced more rapidly than the same mixture at pH 4.4 (Fig. 1.). It is well known that in lower pH reducing environments, iron is reduced more rapidly and this has been attributed to greater solubility of iron at a low pH (Goodell et al., 1997). In the presence of 2,3 DHBA, the same interpretation can be made for the accelerated iron reduction at a lower pH.

Fig. 1. Comparison of FeII generation in the presence of 2,3 DHBA and with, or without oxalate at pH conditions of 2.2 (a) or 4.4 (b). Final reaction mixtures contained 30μM FeIII, 2μM 2,3-DHBA and 250μM Ferrozine reagent. 45μM oxalate was added for the oxalate-containing samples.

FeCl₃ that was pre-incubated with oxalate was reduced less rapidly in the presence of 2,3-DHBA than the samples without oxalate. This was observed at both pHs, and has been previously attributed to the formation of stable Fe-Oxalate complexes that impede iron reduction (Arantes et al., 2009). However, in the oxalate-containing samples, iron reduction occurred more slowly, and less iron overall was reduced at pH 2.2 compared to pH 4.4 (Fig. 1), indicating that the pH 4.4 environment was more favorable for iron transfer from oxalate to 2,3-DHBA. Similar findings when oxidized iron was incubated with oxalate were previously observed (Goodell et al. 1997). This data suggests why the pH differential between the fungal extracellular matrix (ECM) and the wood cell wall, would help to promote iron reduction within the wood cell wall where redox reactions generated by the CMF will promote cell wall deconstruction.

3.2 ROS production with 2,3-DHBA oxalate-sequestered iron and detection by EPR spin-trap spectroscopy

EPR spin trapping studies showed that ROS were generated when all species of iron tested were mixed with 2,3 DHBA and H₂O₂ (Fig. 2). DMPO will trap both hydroxyl radicals (the four dominant peaks) as well as superoxide radicals (the smaller peaks between the four dominant

peaks). However at both pH 2.2 and 4.4, samples containing Fe(Oxy)-only displayed a reduced ROS signal as compared to samples with Fe(Fre). This indicates that the oxygenated iron was less able to participate in CMF reactions. In this regard, the lack of available iron is well known in most microbial systems, and for this reason many organisms produce chelators to sequester insoluble iron from the environment (Kraemer, 2004; Neilands, 1995; Schwyn and Neilands, 1987). Prior research has demonstrated that brown rot fungi preferentially produce more catecholate chelators which are capable of sequestering and reducing iron, whereas white rot and other fungi produce other types of chelators such as hydroxamic acids which do not readily reduce iron (Goodell et al., 2006; Goodell et al., 2002; Qi, 2001). However, it must be recognized that free ferrous iron would not typically be available in fungal or soil environments. In the fungal decay environment therefore, the low pH reduction - which requires soluble iron, would therefore not occur.

Fig. 2. EPR spin-trap spectra when different iron (30 μ M) species including Fe(Fre), Fe(Oxy)-Oxalate, Fe(Oxy) were mixed in the presence of 2,3-DHBA (30 μ M) and H₂O₂ (1.5mM) at pH 2.2 (a) and 4.4 (b).

An increased ROS signal was observed by pre-incubating Fe(Oxy) with oxalate and then mixing with 2,3-DHBA and H₂O₂ (Fig. 2). As oxalate is typically produced in early stages of wood decay by the fungi, the sequestration of iron by oxalate from the environment would be expected to occur. This study confirmed that oxalate was able to promote enhanced CMF reactions when iron was sequestered prior to CMF redox reactions. When using oxalate-sequestered oxide-hydroxide iron [Fe(Oxy)-Oxalate], reactions at a higher pH yielded greater activity in the CMF reaction. This is because the higher pH (4.4) was more favorable for the iron transfer from oxalate to 2,3-DHBA as discussed above. In the fungal/wood environment, reactions favoring ROS generation would therefore be favored in the higher pH environment of the wood cell wall as opposed to the environment directly surrounding the fungal hyphae and within the ECM.

3.3 Iron sequestration by oxalate

FeII production was monitored in the presence of oxalate and 2,3-DHBA under conditions dependent on both pH and the concentration of oxalate.

pH 2.0: At pH 2.0, iron reduction by 2,3-DHBA in Fe(Oxy) samples was initially greater in the absence of oxalate (Fig. 3a). Only over time (during the 24 hour analysis) was it apparent that oxalate solubilized the iron and made more of it available for reduction by 2,3-DHBA. This was most apparent at the lowest concentration of oxalate tested (15 μ M). Intuitively, a higher concentration of oxalate will sequester more iron (Ambikadevi and Lalithambika, 2000). However, with increasing concentration of oxalate, even though more iron would have been solubilized, transfer of iron to 2,3-DHBA was reduced, and thus less reduction of iron occurred over time. Therefore oxalate regulation by the fungus, and reduced concentrations which would occur with diffusion into the wood cell wall, would likely play important roles in maintaining appropriate levels of oxalate to permit the transfer of iron to catecholate chelators in the decay environment.

Siderophores and other chelators produced by microorganisms have a high affinity and

specificity for iron binding and have been reported to assist in iron acquisition from iron oxides (Boukhalfa and Crumbliss, 2002; Kraemer, 2004). However, chelators like 2,3-DHBA require additional chelators like oxalate to sequester iron more efficiently in pH 2.0 conditions. In this regard, we suggest that pH reduction is less important for iron sequestration by brown rot fungi, and secretion of oxalate or other compounds by brown-rot fungi is necessary for the solubilization of Fe(Oxy) forms in the environment. Ligand adsorption, metal detachment and proton adsorption (Stumm and Furrer, 1987) all play a role in these reactions. However, it is apparent, as demonstrated in section 3.1 and 3.2 that control of pH and the generation of a pH differential can aid in the generation of ROS in appropriate locations so that less damage may occur to the fungal hyphae and ROS can be generated within the wood cell wall.

Fig. 3. Kinetic iron sequestration by oxalate over a range of concentrations, including 15 μ M [Fe(Oxy)-Oxa(15 μ M)], 30 μ M [Fe(Oxy)-Oxa(30 μ M)], and 120 μ M [Fe(Oxy)-Oxa(120 μ M)]. Reactions were run at pH 2.0 (a), pH 3.5 (b) and pH 4.8 (c).

pH 3.5 and 4.8: Although similar trends relative to the role of oxalate in solubilizing iron at pH 2.0 was observed at the higher pHs tested, iron reduction was less at pH 3.5 (Fig. 3b.) and lesser still at pH 4.8 (Fig. 3c). It is well known that lower pH environments function as reducing environments, and this data confirms this. The presence of two different types of chelators, which is required in CMF reactions, complicates the findings and so it is useful to separate out the roles of the two types of chelators. Even though lesser amounts of iron are reduced overall at a higher pH, and this would also be expected to occur in the wood cell wall, it is important to recognize the reduction of large amounts of iron may not be necessary to initiate CMF deconstruction of the wood cell wall. The more critical issue is a mechanism to allow the abundant, but unavailable, Fe(Oxy) forms of iron in the environment to be accessed by the CMF fungal mechanism. The production of oxalate permits this to occur, but it does not generate the reduced iron necessary for Fenton reactions. Only catecholate chelators permit this reduction to initiate ROS generation. As demonstrated in Fig. 1 and 2, these reactions proceed in the presence of oxalate. Therefore, as long as limited amounts of iron can be sequestered by oxalate at the lower pHs around the fungal hyphae and in the aqueous environment of the ECM, when the oxalate-sequestered iron diffuses into a higher pH environment, and in the presence of a catecholate chelator like 2,3-DHBA, iron reduction will occur. Redox cycling of the iron will then permit multiple moles of ROS species to be generated for each mole of catecholate chelator produced (Pracht et al., 2001; Qian et al., 2002).

These data confirm the necessity of oxalate control by the fungus (Connolly and Jellison, 1995; Espejo and Agosin, 1991; Schilling and Jellison, 2005) and suggest that further research is needed in this area, but also on earlier reports indicating that control of oxalate concentration may be controlled not only by oxalate secretion, but also by the solubilization and recrystallization of oxalate on the fungal hyphae surface and within the ECM (Connolly and Jellison, 1995; Zhuang et al., 2015).

4. Conclusion

Oxalate-sequestered iron was reduced by the chelator (2,3-DHBA) permitting the generation of $\cdot$ OH in the presence of H₂O₂. The presence of oxalate suppressed, but did not stop

iron reduction, although the amount of iron reduction by 2,3-DHBA was dependent upon the oxalate concentration. Both pH and oxalate concentration played an important role in sequestering iron from oxidized iron for subsequent Fenton reactions. Our data suggest that the secretion of oxalate by brown-rot fungi is essential to making insoluble and unavailable forms of iron in the environment available for the non-enzymatic degradation via the CMF reaction. Iron sequestration by oxalate was enhanced at lower pH environments, but oxalate concentration appeared to play a greater role in overall iron reduction, and may also do so in brown-rot decay mechanisms. These results suggest that the secretion of oxalate by brown-rot fungi enhances the CMF reaction in early stages of brown-rot decay. These findings provide evidence for the previous theory of oxalate regulation by decay fungi, including oxalate biosynthesis and its metabolism (Evans, 1994; Schilling, 2006; Schilling and Jellison, 2005).

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Fig. 3. Kinetic iron sequestration by oxalate over a range of concentrations, including 15 μ M [Fe(Oxy)-Oxa(15 μ M)], 30 μ M [Fe(Oxy)-Oxa(30 μ M)], and 120 μ M [Fe(Oxy)-Oxa(120 μ M)]. Reactions were run at pH 2.0 (a), pH 3.5 (b) and pH 4.8 (c).

Figure 1

Figure 2

Figure 3

Table 1. Samples for iron sequestration by a kinetic Ferrozine assay. Three 100mM buffer solutions were used: pH 2.0 (Clark and Lubs buffer) and 3.5 and 4.8 (both acetate buffers) and all samples were made up to 3 ml.

SAMPLE	Iron (μM)		Oxalate (μM)	2,3-DHBA (μM)	Ferrozine (μM)
	Fe(Fre)	Fe(Oxy)			
Fe(Oxy)	—	30	0	30	250
Fe(Oxy)-Oxa (15 μM)	—	30	15	30	250
Fe(Oxy)-Oxa (30 μM)	—	30	30	30	250
Fe(Oxy)-Oxa (120 μM)	—	30	120	30	250
Fe(Fre)	30	—	0	30	250