Inhibition of Cellulase-catalyzed Lignocellulosic Hydrolysis by Iron and Oxidative Metal Ions and Complexes

Ani Tejirian, Feng Xu*

Novozymes, Inc., Davis, CA 95618, United States of America

* Corresponding author. Mailing address: Novozymes, Inc., 1445 Drew Avenue, Davis, CA 95618, United States of America. Phone: (530) 757-8100. Fax: (530) 758-0317. Email: fxu@novozymes.com.

Keyword: oxidative metal ion, cellulase, inhibition, cellulose hydrolysis, biomass conversion

Running title: Inhibition of cellulase reaction by oxidative metal ions
Abstract

Enzymatic lignocellulose hydrolysis plays a key role in microbially-driven carbon cycling and energy conversion, and holds promise for bio-based energy and chemical industries. Cellulases (key lignocellulose-active enzymes) are prone to interference from various non-cellulosic substances (e.g., metal ions). During natural cellulolysis, these substances may arise from other microbial activities or abiotic events, and during industrial cellulolysis, they may be derived from biomass feedstocks or upstream treatments. Knowledge about cellulolysis-inhibiting reactions is of importance for the microbiology of natural biomass degradation and the development of biomass conversion technology. Different metal ions, including those native to microbial activity or employed for biomass pretreatments, are often tested in enzymatic cellulolysis. Only a few metal ions act as inhibitor for cellulases, which include ferrous and ferric ions as well as cupric ion. In this study, we showed that the inhibition from ferrous/ferric ions as part of a more general effect from oxidative (or redox-active) metal ions and their complexes. The correlation between inhibition and oxidation potential indicated the oxidative nature of the inhibition, and the dependence on air established the catalytic role iron ions played in mediating the dioxygen inhibition on cellulolysis. Individual cellulases showed different susceptibilities toward the inhibition. Likely the inhibition exerted its effect more on cellulose than on cellulase. Strong iron ion chelators and polyethylene glycols could mitigate the inhibition. Potential microbiological and industrial implications of the observed effect on enzymatic cellulolysis from redox-active metal ions, as well as prevention and mitigation of this effect in industrial biomass conversion, were discussed.
Microbial degradation of lignocellulosic biomass (mostly derived from plant cell walls) is important for carbon cycling, energy conversion, microbiota vitality, animal-microbe symbiosis, and other microbiological or ecological processes. In general, the biodegradation of lignocellulose takes place in highly heterogeneous environments (e.g., soil, sediment, swamp, marsh, or animal digestive track), in which different microbes employ different biological ways (of lytic, redox, synthetic, or other nature) to seek nutrients, compete with others, or fight off inactivation. Lignocellulolytic microbes use an array of specific hydrolases, lyases, oxidoreductases, and accessory enzymes to constitute enzymatic machineries able to effectively degrade or convert highly complex lignocellulosic substances (1, 23). In nature, these enzymes have to have not only high specificity and reactivity toward their substrates (targeted lignocellulosic substances), but also resilience toward inhibitors or inactivators derived from other biomass-targeting enzymatic reactions as well as other co-existing microbial or abiotic processes. Potential impact from redox chemistry, one of the important ecological or environmental properties for many microbiotas, might become significant because of two sets of microbial activities.

First, many microbes employ oxidoreductase-derived redox agents to degrade or modify lignin, a major plant cell wall substance that not only coexists with but also protects cellulose (from degradation by foreign agents). For instance, white rots secrete diffusible heme-containing peroxidases (lignin peroxidase, Mn peroxidase, versatile peroxidase) to attack lignin, supported by H$_2$O$_2$ generated by secreted carbohydrate or alcohol oxidases, and assisted by radicalized surface amino acid residues, dissociated Mn(III), or separate redox mediators (for recent reviews, see 33). Brown rots secrete redox-active quinoids or Fe-chelating peptides, H$_2$O$_2$-generating carbohydrate or alcohol oxidase, and other oxidoreductases (including laccase), to modify lignin and degrade cellulose by OH radicals from a Fenton-type chemistry (for recent reports, see 1, 42). These oxidoreductases and their redox metabolites, products, or mediators (e.g., Mn(III), H$_2$O$_2$) might act on or affect cellulases (secreted by the same or different lignocellulolytic microbes) or their lignocellulose hydrolysis.

Secondly, all microbial life cycles depend on redox chemistry, and many general microbial processes are of redox nature. Based on their final electron acceptors (or cellular respiration modes), microbes can be grouped as aerobes (able to reduce O$_2$ to H$_2$O), nitrate reducer or denitrifier (able to reduce NO$_3^-$ to N$_2$), Mn(IV) reducers (able to reduce MnO$_2$ to Mn(II)), Fe(III) reducers (able to reduce Fe$_2$O$_3$ or
FeO(OH) to Fe(II)), sulfate reducers (able to reduce SO$_4^{2-}$ or S to HS$^-$), or methanogens (able to reduce CO$_2$ to CH$_4$). Among them, the Mn(IV) and Fe(III) reducers, as well as general anoxygenic photosynthesis, can generate diffusible redox-active mediators and Mn or Fe species. Spatial and temporal distribution of different microbes or respiration modes, and consequently redox environments or abundance of redox-active agents, in microbiota is subjected to air O$_2$ availability and geochemical conditions (for recent reviews, see 4, 34). When exposed to air, aerobic cellulolytic microbes (mostly fungi) degrade cellulose by coupling the process to their respiration. Local anoxicity/anaerobicity may be formed due to fast metabolisms by these and other aerobes, thus allowing anaerobic cellulolytic microbes (mostly bacteria) to degrade cellulose. Aerobic cellulolytic microbes may border with, and anaerobic cellulolytic microbes may live within, non-cellulolytic anaerobes (facultative or obligate, saccharolytic or syntrophic) relying on non-O$_2$ respirations. Consequently, metabolites or products (including redox-active agents) from the non-cellulolytic anaerobes might contact and affect cellulolytic microbes, their cellulases, or targeted lignocelluloses, as part of a more general cohabitation, collaboration, or competition among different cellulolytic and non-cellulolytic species (for recent reviews, see 23).

Biomass-active microbes and enzymes, as well as natural microbial biomass degradation processes, are highly attractive for the emerging biomass conversion/utilization industry (biorefinery). Three of the major challenges faced by the biomass conversion research and development efforts are the general recalcitrance of biomass, heterogeneity of common feedstocks, and inhibition of specific transformation reactions by coexisting non-substrate substances. In general, the structural and compositional complexity of biomass makes the separation of its components and subsequent transformation to valuable chemicals difficult. For effectively and efficiently converting cellulose (one of the main targets of biomass conversion), one needs to not only optimize the efficacy of cellulases (cellulolytic biocatalysts), but also minimize the detrimental effects from various cellulose derivatives and non-cellulosic substances originated from biomass feedstock or upstream treatments (for recent reviews, see 11, 13, 22, 42, 44).

Biomass (as either direct substrate for natural microbial biomass degradation or feedstock for industrial bioalcohol and other chemical productions) are in general highly heterogeneous and complex, comprising cellulose, hemicellulose, lignin, protein, extractive, mineral, and other substances. Although
carbohydrates are the main targets for developing biomass-active enzymes, other minor biomass components are of interest as well, because they may affect the activity of biomass-active enzymes. Metal ions could be such effectors, since they exist universally in microbiota habitats (e.g. Ca(II), Al(III), and other metal ions adsorbed in soil or dissolved in water; Fe(III), Fe(II), Mn(IV), and Mn(II) from anaerobes’ respirations and anoxygenic photosynthesis), and often serve as structural/functional cofactors (e.g. Ca(II), Zn(II), Fe(II), Mn(II), Cu(II)) or inhibitors (e.g. Ag(I), Hg(II), Pb(II)) of many enzymes including cellulases. In corn stover, a major feedstock, iron oxide may account for 0.5% of the ash, and Mn may be present at 23 mg/kg (25). In nature, biomass-degrading microbial enzymes (e.g. cellulases, hemicellulases) are most likely in contact with various metal ions (including redox-active ones) related to either other enzymatic, microbial, or abiotic processes. Some metal ions may also be present at significant levels in industrial enzymatic biomass-converting systems, after being added as reagents, dissolved from reactor/pipeline, or brought from water or other raw material sources.

As part of cellulase characterization, common divalent metal ions (e.g. Mg(II), Ca(II), Mn(II), Fe(II), Co(II), Ni(II), Cu(II), Zn(II), and Hg(II)) as well as mono- or trivalent metal ions (e.g. Na(I), K(I), and Fe(III)) are often tested for their effect on cellulase activity (24). In general, Hg(II) behaves as a fatal cellulase inhibitor, likely because of its interaction with key sulfur-containing amino acid residues (as it does on other proteins). Among the divalent metal ions, most often only Cu(II) and Fe(II) are found exerting strong inhibition on cellulose-hydrolyzing reactions of cellobiohydrolase (CBH), endo-β-glucanase (EG), or β-glucosidase (BG) (for examples, see 12, 15, 30). The cellulase-inhibiting actions of Fe(II) and Cu(II), but not Mn(II), Co(II), Ni(II), and Zn(II) (all have the same +2 charge and similar size), have not been well understood from mechanistic aspect. In general, the effect on cellulases from redox-active metal ions or other compounds during natural microbial biomass degradation has not been extensively investigated.

To further probe the effect of Fe(II) and Cu(II), we carried out a study to exam the hypothesis that these metal ions’ inhibition on cellulases’ reaction is of redox nature. Various redox-active metal ions and complexes were tested comparatively, and possible correlation between these substances’ redox and inhibitory properties was sought. Potential ecological or environmental significance of the redox effect on cellulase’s action is discussed.
MATERIALS AND METHODS

Materials and instruments. Chemicals used as reagents or buffers were commercial products of reagent or purer grade unless specified otherwise. A diluted acid-pretreated corn stover (PCS) preparation (~59% glycan and 28% lignin) kindly provided by United States National Renewable Energy Laboratory, was first ground and sieved, extensively washed by water, then pH-adjusted to ~5. Carboxymethyl cellulose (CMC) was from Hercules (7L2 type, 70% substitution). Phosphoric acid-swollen cellulose (PASC) was prepared from Avicel (FMC, PH101) by a published method (35). Fe-(2,2'-bipyridyl)$_2$ complex was prepared by mixing 0.1 M FeCl$_2$ or FeCl$_3$ with 0.2 M 2,2'-bipyridyl (2,2'BP) in water at 23°C. Fe-(1,10-phenanthroline)$_2$ complex was prepared by mixing 0.1 M FeCl$_2$ or FeCl$_3$ with 0.2 M 1,10-phenanthroline in water. Fe-EDTA complex was prepared by mixing 0.1 M FeCl$_2$ or FeCl$_3$ with 0.1 M EDTA in water. An experimental cellulase preparation from Novozymes (“cellulase mix”) was used as cellulase mixtures for cellulose hydrolysis (cellulolysis). The mix contained extracellular Hypocrea jecorina (Trichoderma reesei) cellulates and Aspergillus oryzae CEL3A BG. Wild-type H. jecorina CEL7A CBH-I, recombinant H. jecorina CEL6A CBH-II, CEL7B EG-I, and CEL5A EG-II, as well as recombinant CEL3A, were either prepared as previously reported (14, 45, 46).

Spectrophotometric measurement was carried out on a Molecular Devices SpectraMax 340PC or Gemini XPS reader with Costar 96-well microplates. Sugar analysis was carried out on either an Agilent 1100 HPLC instrument, equipped with a Bio-Rad Aminex HPX-87H column and a refractive index detector, under 5 mM H$_2$SO$_4$ elution. Temperature-controlled incubations were made in New Brunswick Scientific Innova 4080 incubation shakers. Polyacrylamide electrophoresis (PAGE) was carried out in a Bio-Rad Criterion cell with Criterion precast gels, and stained with Bio-Rad’s Bio-Safe Coomassie kit.

Enzymatic cellulose hydrolysis. PCS hydrolysis was carried out in either 1.7-mL Eppendorf microcentrifuge tubes or 2-mL 96-well VWR Deep Well plate, typically with 43.4 g/L PCS (dry weight), 0.25 g/L (~4 μM based on an average molecular weight (MW) of 60 kD) cellulase mix, 50 mM Na-acetate, pH 5, in 1 mL suspensions and at 50°C for up to 4 days, under 150 rpm shaking. Heat-sealing of the plate was made by an ABgene ALPS-300 device (160°C for 2 s). Aliquots (80 µL) of the suspension were
sampled and then centrifuged or filtered (on Millipore Multiscreen-HV 96-well plate filters), and the
supernatants were analyzed for soluble reducing sugars on HPLC. Hydrolysis extent was estimated from
the observed glucose and cellobiose (longer cellooligomers were negligible), and based on the glycan content
of the PCS, taking into consideration on water incorporation after glucosidic bond cleavage. Initial rate
was obtained from the hydrolysis during the first day. Hydrolysis of PASC, Avicel, and cellobiose was
carried out under conditions similar to that of PCS, and their typical dosing was 2, 23, and 2 g/L level,
respectively. Typical cellulase dosing was 0.25 g/L cellulase mix or 0.04 g/L (or ~0.7 μM) individual
cellulase. For 50-μL scale hydrolysis, the enzyme/substrate levels were scaled up accordingly, and
hydrolysis was carried out in rubber-stopped 125-mL Erlenmeyer flasks. CMC hydrolysis was made in 1
mL solutions with 10 or 20 g/L CMC, 1-20 mg/L cellulase, and 50 mM Na-acetate, at pH 5 and 50°C.
Aliquots of the suspension were sampled, and their supernatants were analyzed for soluble reducing sugars
by p-hydroxybenzoic acid hydrazide (PHBAH), based on a published method (16). Background absorption
was corrected by control reactions.

When anaerobicity was needed, 125-mL Erlenmeyer flasks with glass stopper (Chemglass CG-
8506) and an inflatable/sealed/gas-controlled chamber (Aldrich AtmosBag) were used. The chamber was
filled with N₂ (Airgas, 99.9%), and pre-made cellulose suspension was deaerated by gentle bubbling with
N₂ (for 2 min). The suspension and enzyme stock were mixed, and the flasks were sealed inside the
chamber. Temporal sampling of the hydrolysis was made inside deaerated chamber. The glass stoppers
provided lasting anaerobicity, although they needed to be held tightly against increased (~10% higher)
internal gas pressure (when the sealed flasks were warmed up from 23 to 50°C) by copper wires anchored
around the flask necks. Hydrolysis suspensions assembled/sampled in open air served as controls.

At least duplicates were run in each experiment.

Inhibition from metal ions or complexes. Evaluation of selected metal ions for their effect on
enzymatic cellolysis was exemplified by that of Fe(II). Stock solutions of Fe(II) were prepared at 0.25 M
as FeSO₄ in water, unless specified otherwise. FeSO₄ was added to the cellulose hydrolysis suspension and
their inhibitions were evaluated by the decrease of hydrolysis. For PASC hydrolysis, 0.6 to 4 g/L PASC,
0.01 g/L cellulase mix, and 0.1 to 10 mM FeSO₄ were reacted in 50 mM Na-acetate of pH 5. As a control,
FeSO₄ were incubated with PASC and no hydrolysis was seen. No effect from FeSO₄ on the HPLC
profiles of glucose and cellobiose was seen, either. For Avicel hydrolysis, 0.6 to 4 g/L Avicel, cellulase
mix, and 0.5 to 4 mM FeSO₄ were reacted in 50 mM Na-acetate of pH 5. For Avicel hydrolysis, 0.6 to 4
g/L Avicel, 0.25 g/L cellulase mix, and 0.5 to 4 mM FeSO₄ (or 0.2 to 2 mM other selected redox-active
ions/complexes) were reacted in 50 mM Na-acetate of pH 5. For PCS hydrolysis, 43.4 g/L PCS, 0.25 g/L
cellulase mix, and 0.1 to 10 mM FeSO₄ were reacted in 50 mM Na-acetate of pH 5. For individual
cellulases, Fe(II) inhibition was tested similarly, with 0.6 to 4 g/L PASC, 0.04 g/L CEL7A or other
cellulases, and 3 to 15 mM FeSO₄, for up to 4 h incubation. For CEL3A, cellobiose was used instead of
PASC.

Initial hydrolysis rate was obtained from the first two hydrolysis time points (with < 20% hydrolysis extent in general, rate = (hydrolysis difference)/(time difference)). Double-reciprocal plots
(1/(initial rate) vs 1/[cellulose] as function of [inhibitor]) were employed to study inhibition types and
derive inhibition constant $K_i$ when possible. Initial rate vs [inhibitor] plots (linear interpolation), as
function of [cellulose], were used to derive $I_{50}$, the inhibitor concentration resulting in 50% loss in initial rate.

Mitigation of iron ion inhibition on enzymatic cellulolysis. Potential mitigators of iron ion
inhibition were tested in PCS hydrolysis. In one experiment, the hydrolysis of 43 g/L PCS by 0.25 g/L
 cellulase mix, in 50 mM Na-acetate of pH 5 at 50°C, was monitored with 2.5 mM FeSO₄, 10 mM H₂O₂,
and 10 mM desferrioxamine (siderophore), added either alone or in combination after 30 min pre-
incubation prior to cellulases addition. In another experiment of the PCS hydrolysis, 10 mM
desferrioxamine, 1,10-phenanthroline, or 2,2'-bipyridyl was added so the effect of the Fe(II) chelators on
the hydrolysis could be evaluated.

Potential effect from polyethylene glycol (PEG) on Fe(II)-caused inhibition of enzymatic
cellulolysis was studied with PEG4000. In one experiment, 10 mM FeSO₄ and 0.625 to 5 g/L PEG4000
were added either alone or in combination to PCS hydrolysis with cellulase mix. In another experiment, 10
mM FeSO₄ and 5 g/L PEG4000 were added to Avicel hydrolysis with cellulase mix. The effect of 50 g/L
PEG4000 on the inhibition by 10 mM FeSO₄ on the hydrolysis of either 50 g/L PCS or 25 g/L Avicel by
0.25 g/L cellulase mix was also compared.
Pre-incubation of celluloses or cellulose with inhibitory iron species. To pre-incubate Avicel or PASC with inhibitory iron species, prior to hydrolysis by cellulase mix, 25 g/L Avicel or 2 g/L PASC was mixed with 10 mM FeSO₄, FeCl₂, or FeCl₃ in water at 23°C for 2 h. Pre-treated cellulose was then extensively washed with 50 mM Na-acetate of pH 5 (until the final Fe species in supernatant reached <0.05 mM), and hydrolyzed with cellulase mix. To pre-incubate cellulases with inhibitory iron species, prior to the enzymes cellulolysis, 2.5 (for Avicel hydrolysis) or 0.01 (for PASC hydrolysis) g/L cellulase mix was mixed with 10 mM FeSO₄, FeCl₂, or FeCl₃ in buffer at 23°C for 2 h or 3 d. Pre-treated cellulases were then desalted on Bio-Rad’s Bio-Spin 6 columns (to remove iron species), and applied to hydrolyze Avicel or PASC. Hydrolyses of untreated or buffer-only pre-incubated Avicel, PASC, and cellulase mix, with or without iron inhibitors, served as controls.

Other assays. Protein determination of cellulase samples was made with Pierce BCA kit after subjecting samples to gel-filtration.

Fe ion quantification by 2,2’-bipyridyl was carried out by mixing Fe(II) or Fe(III)-containing samples with 5 mM 2,2’-bipyridyl, and measuring electronic absorption at 520 nm. Solutions of 0.005 to 0.1 mM FeSO₄ or FeCl₃ were used for calibration. At same concentration, Fe(III)(2,2’BP)₃’s absorption was negligible comparing to that of Fe(II)(2,2’BP)₃.

Possible Fe ion adsorption onto cellulose was detected by incubating 5 to 100 µM FeSO₄ with 14 to 25 g/L Avicel in 50 mM Na-acetate of pH 5 (1 mL suspension) at 50°C. After 1 to 24 h, the supernatants were subjected to 2,2’-bipyridyl quantification.

Possible Fe(II) effect on cellulases was examined by non-denaturing polyacrylamide gel electrophoresis (PAGE). Cellulase mix (2.5 g/L) or individual cellulases were first incubated with 10 mM FeSO₄ in 50 mM Na acetate of pH 5 at 50°C for 4 days. The samples were then subjected to non-denaturing (SDS-free, native PAGE sample buffer, Tris-glycine running buffer) PAGE on 8-16% gels. Fresh cellulase mix and individual cellulases were also run as controls.

To strip away potential accessible Fe ion in PCS, washed PCS was incubated with 10 mM 2,2’-bipyridyl, 1,10-phenanthroline, or 1,7-phenanthroline for two days, and then washed extensively to remove the chelator or Fe-chelator complex. The treated PCS was then subjected to the hydrolysis with selected cellulases.
RESULTS

Inhibitory effect of Fe(II) addition on enzymatic cellulolysis. When several metal cations, as either chloride or sulfate salt, were added (individually) to the enzymatic hydrolysis of PCS, only FeSO₄ and FeCl₂ caused a significant inhibition of the hydrolysis (Fig. 1). Since other tested sulfates and chlorides were benign, the inhibition was most likely due to Fe(II).

The concentration dependence of Fe(II)’s inhibition on enzymatic cellulolysis was probed with the hydrolysis of Avicel or PASC by cellulase mix. For the hydrolysis PASC by cellulase mix, the addition of FeSO₄ resulted in 1/(Initial rate) vs 1/[PASC] plots (as function of [FeSO₄]) indicative of a mix-type inhibition, whose complexity prevented extraction of simple Kᵢ. For the hydrolysis of Avicel by cellulase mix, the addition of FeSO₄ also resulted in double-reciprocal plots indicative of a mix-type inhibition, but no simple Kᵢ could be extracted due to plot complexity. Table 1 shows the Iₛₒ obtained from Initial rate vs [FeSO₄] plots. Apparently, Fe(II)’s inhibition on Avicel hydrolysis was more pronounced than that on PASC. For the hydrolysis of PCS, an Iₛₒ ~7 mM was observed for FeSO₄.

Fe(II) also exerted inhibition on PASC hydrolysis by the major components of cellulase mix (Fig. 2). For CEL7A, CEL7B, CEL5A, and CEL3A, the Initial rate vs [Fe(II)] plots appeared as mixed type, whose complexity also prevented extraction of simple Kᵢ. Table 1 shows the Iₛₒ obtained from Initial rate vs [FeSO₄] plots. Apparently Fe(II) exerted a more pronounced inhibition on CEL7B’s reaction than those on CEL7A and CEL5A. For CEL3A’s reaction, Fe(II)’s inhibition seemed minor. CMC hydrolysis was also tested for Fe(II)’s inhibition on CEL7B and 5A (both EGs), but the PHBAH assay was significantly interfered by Fe(II), making the reducing sugar measurement highly variable.

Differential inhibition of enzymatic cellulolysis by redox-active metal ions. To probe whether or not Fe(II)’s inhibitory effect is related to its ionic properties (size and charge), various (biologically common) divalent metal ions with variable ionic radii were tested in Avicel hydrolysis by cellulase mix. At 10 mM level, MgCl₂ and CaCl₂ showed slight enhancement, while CoSO₄, MnSO₄, NiCl₂, and ZnSO₄ showed slight or moderate inhibition, on the hydrolysis. In contrast, FeSO₄ (as well as FeCl₂), and CuSO₄ resulted in ~70% or more loss of both initial hydrolysis rate and hydrolysis extent after 4 days (Table 2).
MnSO$_4$ was also tested at 0.1 to 10 mM range, and no effect on the hydrolysis was observed, although FeSO$_4$ at the same range caused a concentration-dependent inhibition. Because of its susceptibility to O$_2$ oxidation, initial added Fe(II) was steadily transformed to Fe(III), as indicated by a brown color appearance of the hydrolysis suspension. To test whether Fe(II) exerted its inhibition via air-oxidized Fe(III), a series of 50-mL scale hydrolysis of 25 g/L Avicel by 0.25 g/L cellulase mix with or without 1 mM FeSO$_4$, in 50 mM Na acetate pH 5 at 50°C, was carried out in “normal” (aerated) or N$_2$-deaerated solutions. After one day, FeSO$_4$ caused ~30% decrease in the “aerobic” cellulolysis, but no effect in the “anaerobic” cellulolysis, indicating the involvement of O$_2$ in Fe(II)’s apparent inhibition on cellulolysis.

The effect of adding H$_2$O$_2$ to Fe(II)-inhibited PCS hydrolysis was studied. For the hydrolysis of 43.5 g/L PCS with 0.25 g/L cellulase mix with or without 10 mM FeSO$_4$, 0, 1.25, 2.5, 5, and 10 mM H$_2$O$_2$ were added. No change on the inhibition was seen, indicating that it was the Fe species, rather than oxygen-based species derivable from Fenton chemistry, that caused the inhibition mainly.

To directly study Fe(III)’s effect on enzymatic cellulolysis, FeCl$_3$ was added into Avicel hydrolysis by cellulase mix. At 10 mM level, FeCl$_3$ resulted in ~90% loss of both initial hydrolysis rate and hydrolysis extent after 4 days (Table 2). In a hydrolysis of 2 g/L PASC by 0.01 g/L cellulase mix, 10 mM FeCl$_3$ resulted in ~80% loss of both initial hydrolysis rate and hydrolysis extent after 4 days, comparing to the ~30% loss brought by FeCl$_2$. Thus, Fe(III) exerted more potent inhibition than Fe(II).

In addition to Fe(III), two other trivalent and oxidative metal ions, Cr(III) and Ru(III), were also tested. At 10 mM level, CrCl$_3$ and RuCl$_3$ exerted moderate and pronounced inhibition on enzymatic Avicel hydrolysis, respectively (Table 2).

**Differential inhibition of enzymatic cellulolysis by iron-chelator complexes.** To further probe how iron ions inhibited enzymatic cellulolysis, iron-chelator complexes with variable size or redox property were tested. For Avicel hydrolysis by cellulase mix, 10 mM Fe(2,2’-BP)$_3$Cl$_3$, FeCl$_3$, FeCl$_2$, Fe(2,2’-BP)$_3$Cl$_2$, FeNa(EDTA), K$_3$Fe(CN)$_6$, K$_4$Fe(CN)$_6$, or Fe-citrate exerted various degrees of inhibition (Table 3). Iron complexes with higher oxidation potential $E^*$ tended to cause more hydrolysis inhibition (Fig. 3).
Concentration dependence of the effect of inhibitory metal ions (in addition to Fe(II)) and complexes was also studied in Avicel hydrolysis by cellulase mix. As shown in Table 4, oxidative Fe(III), Ru(III), and Cu(II) species had $I_{50}$ much lower than Fe(II) species.

To further probe the connection between $E^\circ$ and cellulolysis inhibition of oxidative substances, $p$-benzoquinone and $p$-hydroquinone (reduced quinone), with an $E^\circ$ of 0.715 V (10), was tested in Avicel hydrolysis by cellulase mix. At 5 mM level, quinone resulted in ~50% loss in both initial hydrolysis rate and extended hydrolysis extent, while $p$-hydroquinone exerted no effect.

Differential targeting of iron ion’s inhibition on cellulase and cellulose. To test whether Fe(II) affect cellulose or cellulase, FeCl$_2$ was pre-incubated with either Avicel or cellulase mix for three days. “Fe-pretreated” Avicel and cellulase mix were washed and gel filtered, respectively, to remove residual Fe ion. Fe-pretreated Avicel was hydrolyzed with fresh cellulase mix or cellulase mix subjected to “FeCl$_2$-less” pre-incubation and gel filtration. Fe-pretreated cellulase mix was applied to hydrolyze fresh Avicel or Avicel subjected to “FeCl$_2$-less” pre-incubation and washing, in comparison with the hydrolysis of fresh Avicel (2 g/L) by fresh cellulase mix (0.16 g/L), with or without 10 mM FeCl$_2$. Pre-incubating FeCl$_2$ with Avicel or cellulase mix led to ~10 or 20% loss, respectively, in both hydrolysis initial rate and extended extent. Fresh FeCl$_2$ led to ~50% loss in both hydrolysis initial rate and extended extent for the hydrolysis of fresh Avicel by fresh cellulase mix. Pre-incubating cellulase mix or Avicel with buffer resulted in no effect on hydrolysis.

To test whether Fe(III) affect cellulose or cellulase, FeCl$_3$ was also pre-incubated with either Avicel or cellulase mix for three days. Pre-incubating FeCl$_3$ with Avicel or cellulase mix led to ~80 or 20% loss, respectively, in both hydrolysis initial rate and extended extent. Fresh FeCl$_3$ led to ~90% loss in both hydrolysis initial rate and extended extent for the hydrolysis of fresh Avicel by fresh cellulase mix.

The “pre-incubation” study was also made with the hydrolysis of PASC. Pre-incubating FeCl$_2$ with PASC led to ~60% and no loss, respectively, in initial rate and extended hydrolysis extent. Pre-incubating FeCl$_3$ with cellulase mix led to ~70% and no loss, respectively, in initial rate and extended hydrolysis extent. Fresh FeCl$_2$ led to ~30% and no loss, respectively, in initial rate and extended hydrolysis extent for the hydrolysis of PASC by cellulase mix (fresh or buffer pre-incubated). Replacing FeCl$_2$ with FeSO$_4$ led to similar result. Pre-incubating FeCl$_3$ with PASC led to ~50 and 10% loss, respectively, in...
To probe whether Fe(II) could be adsorbed onto cellulose, a partition study was made. After incubating Avicel and FeSO₄, no detectable Fe(II) adsorption onto Avicel was seen, indicating that Fe(II)’s inhibition on enzymatic cellulolysis was likely not related to cellulose adsorption. The Avicel presence did not significantly affect the auto-oxidation rate of Fe(II) into Fe(III) in aerated solution.

Pre-incubating with FeCl₂ resulted in a PASC whose initial reactivity towards cellulases was inferior to those of fresh or buffer pre-incubated PASC, indicating a detrimental modification by Fe(II) on PASC. Washing FeCl₂-preincubated PASC with 2,2’-bipyridyl, to strip off adsorb iron ions, did not improve the initial reactivity of the PASC towards cellulases, indicating that the Fe-caused PASC modification was not simple Fe adsorption.

Possible interaction between iron ion and cellulase proteins was probed by electrophoresis. On non-denaturing PAGE, in comparison with fresh individual cellulases, the FeSO₄ pre-incubation led to a decrease (or diffusion) of the intensity of the major protein bands in cellulase mix, particularly that of BG. Higher [FeSO₄] in the pre-incubation led to more pronounced decrease in PAGE band intensity. Thus, the interaction with the inhibitors seemed to affect the folding or stability of the major cellulases.

Mitigation of iron ion-caused cellulolysis inhibition. In one experiment, Fe(II) was subjected to oxidation by H₂O₂, and the oxidation effect on the cellulolysis inhibitor was studied. As shown in Fig. 4A, the pre-incubation of FeSO₄, H₂O₂, and PCS, prior to cellulases addition, led to a relief of the inhibition by Fe(II). The presence of desferrioxamine, a strong Fe(III) chelator, resulted in a complete mitigation against the iron ion-caused inhibition on enzymatic cellulolysis. In the absence of Fe(II), H₂O₂ with or without desferrioxamine only affected the hydrolysis slightly (Fig. 4B). At 10 mM, desferrioxamine itself did not affect the hydrolysis of PCS.

At 1 and 10 mM, Fe(III)-citrate did not affect the hydrolysis of PCS by cellulase mix 2, likely due to the Fe(III) chelation by citrate, similar to the case with cellulase mix (Table 3).
Two widely used Fe(II)-chelators, 1,10-phenanthroline and 2,2'-bipyridyl, were tested as potential mitigator for the Fe(II) inhibition on enzymatic cellulolysis. Unexpectedly, the chelators themselves exhibited significant inhibitions on the PCS hydrolysis by NZ cellulose mix: 10 mM 1,10-phenanthroline or 2,2'-bipyridyl resulted in ~40 or 60% decrease in the hydrolysis (in terms of both initial rate and extended hydrolysis extent) of 43 g/L PCS by 0.25 g/L cellulases. In the presence of the chelators, the cellobiose level in the hydrolysis products was about twice as that in the absence of the compounds. Such inhibition by the chelators prevented their use as mitigator against the inhibition of Fe(II) on enzymatic cellulolysis under our conditions.

Polyethylene glycol PEG4000 was tested for potential mitigation of the Fe(II)-caused inhibition of enzymatic cellulolysis. Only insignificant mitigation effect was seen from 5 or 50 g/L PEG4000 on the inhibition of 10 mM FeSO₄ imposed on the hydrolysis of 25 g/L Avicel by 0.25 g/L NZ cellulose mix. For PCS hydrolysis, however, detectable mitigation effect from PEG4000 was seen. For example, 5 g/L PEG4000 apparently fully mitigated the ~20% loss in 0.25 g/L cellulase mix #2's hydrolysis of 43 g/L PCS from 10 mM FeSO₄. However, in the absence of FeSO₄, PEG4000 also enhanced the PCS (but not Avicel) hydrolysis by 5%, an effect reported previously (41, 47). With [PEG4000] varying from 0, 0.625, 1.25, 2.5, to 5 g/L level, PEG4000 showed a dose-dependent mitigation of the Fe(II) inhibition.

The ability of PEG4000 in mitigating Fe(II)'s inhibition could be reduced when H₂O₂ was present. In a hydrolysis experiment of PCS and cellulase mix, the presence of 10 mM H₂O₂ led to a smaller effect from 0.31 g/L PEG against the inhibition of 10 mM FeSO₄, probably indicating a lesser efficacy of PEG4000 against Fe(III) (produced from Fe(II) by H₂O₂).

To test whether there was any significant amount of (accessible) Fe(II) in PCS, and whether removing it with a chelator might help PCS hydrolysis, washed PCS was "stripped" with 10 mM 2,2'-bipyridyl, or 1,10-phenanthroline prior to the hydrolysis by cellulase mix. No detectable effect of the treatment on the hydrolysis was seen, a result that might be attributable to the lacking of a significant amount of Fe(II) in (washed) PCS.

**DISCUSSION**
Apparent inhibition of various cellulases (more precisely, their reactions) by Fe(II) or Cu(II) ion/complexes have been reported (5, 7-9, 12, 15, 17, 18, 26-28, 30, 32, 37). In this study, we also observed significant inhibition from Fe(II) on the major cellulases (CEL7A CBH-I, CEL6A CBH-II, CEL7B EG-I, CELSA EG-II) in H. jecorina, and to a lesser extent on A. oryzae CEL3A BG. A mechanistic understanding of the Fe(II) or Cu(II) effect has not been addressed in previous reports, although it might be ascribed to detrimental binding to cellulases that causes conformational change or replacement of native metal cofactors, or to cellulose that blocks the accessibility for cellulase. Our study suggested that Fe(II) and Cu(II) exert their inhibitory effect on enzymatic cellulolysis via a redox mechanism.

It is known that cellulose can be oxidized, at its “reducing ends”, by Cu(II), and that oxidized cellulose is less reactive than unoxidized cellulose toward cellulase action, including that of CEL6 CBH-II which in general has specificity to cellulose’s non-reducing end (46). Although it has been postulated that the cellulose oxidation might decrease cellulase accessibility, the molecular mechanism of the effect remains to be fully elucidated. Such detrimental oxidative effect is anticipated for oxidants other than Cu(II). In this study we showed that various oxidants (including Fe(II) and Cu(II)), whether as metal ions, organics, or metalloorganics, could inflict significant inhibition on enzymatic cellulolysis (Tables 2-4). Inhibition from Fe(III), Cr(III), and KMnO₄ (a strong oxidant) on cellulase reactions has also been reported before (5, 30, 32, 39). The inhibitory effect depended on E°, the thermodynamic measure of oxidation potency, of the oxidants. Among various ionic compounds, the inhibition effect seemed independent on anions or the charge/size of cations (metal ions), under the conditions of this study (~10 mM level).

In this study, Fe(III) demonstrated a high inhibitory effect (among the tested iron species) on enzymatic cellulolysis. Having an E° of 0.77 V, free Fe(III) (or more likely Fe(III)-acetate (stability constant K: $10^{1.4}$ M⁻¹) in 50 mM Na-acetate buffer) is an oxidant much more potent than Fe(III)-citrate and Fe(III)-desferrioxamine, in which strong chelations by citrate and desferrioxamine greatly stabilize Fe(III) over Fe(II) (K ratio: $10^{10.9}/10^{1.2}$ for citrate, and $10^{30.6}/10^{7.2}$ for desferrioxamine (37, 40)) and consequently lower Fe(III)’s E°. Chelating iron ion with EDTA (K ratio: $10^{25.1}/10^{14.3}$), 2,2’-bipyridyl (cumulative stability constant $\beta_2$ ratio: $10^{6.3}/10^{7.2}$), 1,10-phenanthroline ($\beta_8$ ratio: $10^{14.1}/10^{21}$), and CN⁻ ($\beta_6$ ratio: $
$10^{43.9}/10^{36.9}$ should also affect the oxidation potency of Fe(III) according to the relative stability of chelated Fe(III) and Fe(II) (2, 21, 36).

Because Fe(II) is readily oxidized by air to Fe(III) in aqueous solution, the observed Fe(II)’s inhibitory effect on cellulolysis likely involved Fe(III). Being an oxidant, Fe(III) is known able to oxidize the reducing ends (hemiacetals) of carbohydrates ($E^\circ$: -0.4 V; (10)), while it is reduced to Fe(II) (31). The formed Fe(II) can then be re-oxidized by $O_2$, starting a Fe-catalyzed cycle of cellulose oxidation by $O_2$, in which the Fe shuttles electrons from the reducing ends of cellulose to $O_2$ ($E^\circ$: ~0.9 V for $O_2/H_2O$; (2)). The oxidation can lead to less cellulase-active cellulose and inhibited cellulolysis. Such mechanism was strongly supported by the effect of anaerobicity on Fe(II)’s inhibition, and consistent with the effect of cellulose oxidation by Cu(II) (46).

Pre-incubating cellulases or cellulose with iron ions led to slightly or significantly inactivated cellulases or cellulose, respectively. Likely, iron mainly targeted cellulose for its inhibition on enzymatic cellulolysis. No significant iron ion adsorption onto cellulose seemed to rule out any major role of iron-cellulose complexing in the iron’s inhibitory effect.

Fe(II) showed a stronger inhibition on the hydrolysis of Avicel than that on PASC (Table 1), attributable to the lower accessible reducing ends in Avicel (most of them were within crystalline microfibrils) than those in PASC (swollen, amorphous). Fe(II)’s inhibition on the PASC hydrolysis by cellulase mix seemed to be determined by Fe(II)’s inhibition on both CBH and EG’s actions, based on the $I_{50}$ values (Table 1).

Because of their dependence on $E^\circ$, the inhibitory effect of oxidative (or redox-active) metal ions may be alleviated by lowering $E^\circ$ via differentially complexing their oxidized and/or reduced forms. Chelators strongly favor (thus bind more tightly) the oxidized metal ion form may stabilize it more against the reduced form, leading to lower $E^\circ$ and consequently lower inhibition on enzymatic cellulolysis, as exemplified by Fe(III)-desferrioxamine and Fe(III)-citrate. For Fe(II) and other $O_2$-labile, redox-active enzymatic cellulolysis inhibitors, elimination of $O_2$ (or oxidative conditions) and/or maintenance of a reductive hydrolysis environment may be applied to curtail the substances’ inhibition and regeneration. For practical biomass conversion, citric acid, oxalic acid, EDTA, etc. may serve as low-cost, effective Fe(III) chelators, and CO$_2$, sulfite, etc. may be used for non-oxidative or reductive cellulolysis.
Co-occurring Fe(III)/Fe(II) reactions or other redox events could impact natural lignocellulose hydrolysis by microbial cellulases and hemicellulases, taking place in highly heterogeneous environments in which lignocellulolytic microbes cohabitate with other microbes under diverse aeration, moisture, sunlight, soil composition, and other abiotic or biotic conditions. As part of local microbiota, the growth and action of lignocellulolytic microbes are subjected to the presence and temporal/spatial variations of not only nutrients or substrates, but also inhibitors or regulators, which may include redox-active agents. In addition to (hemi)cellulases, many lignocellulolytic microbes co-secrete oxidoreductases (e.g. peroxidase, laccase) or small redox molecules (e.g. quinone), and generate or utilize environmental redox agents (e.g. H$_2$O$_2$, Fe(III)/Fe(II)), to degrade or modify lignin (1, 33, 42). It remains unclear how a lignocellulolytic microbe regulates its oxidative delignifying or cellulolyzing enzymes or agents to control their potential inhibition on the concomitant hydrolytic cellulase activity, or whether a lignocellulolytic microbe might use its oxidative enzymes/agents to compete with a (lingo)cellulolytic microbe in degrading biomass. Conventionally, microbial enzymatic delignifying systems and cellulolytic systems are studied separately, even when they are part of the whole lignocellulolytic enzyme machinery of the same microbes. Our finding on the oxidative effect on enzymatic cellulolysis calls for more extensive investigation on the interaction between the two systems and its effect on lignocellulose biodegradation.

Redox chemistry not directly related to microbial lignocellulolysis might also impact the biodegradation. Redox-active Fe and Mn species and other diffusible redox mediators are involved in Fe(III)- and Mn(IV)-reducers’ anaerobic respirations and anoxygenic photosynthesis (4, 34). Such redox agents from non-lignocellulolytic co-habitants of local microbiota could affect anaerobic or even aerobic (depending on local O$_2$ distribution) lignocellulolytic microbes and their (hemi)cellulases, either “unintentionally” or competitively. Historically, microbial biodegradation of biomass and lignocellulolytic microbes are studied mostly under simplified laboratory conditions with isolated strains. Our observation of the oxidatively impacted enzymatic cellulolysis suggests that other ecological or environmental factors (including those of redox nature) should be taken into account, so that various “outdoor” lignocellulose biodegradations, as part of either natural carbon cycling, native biota preservation, or artificial soil/water bioremediation, can be further understood.
Variable amount of oxidative or redox-active iron species or other chemicals may exist in industrial biomass hydrolysis reactions, originated from either feedstock, reaction vessel/pipeline/water, or up-stream steps. For instance, a laccase-mediator system has been applied for biomass pretreatment, and the tested N-hydroxy mediators became inhibitory to cellulases after being oxidized by laccase (29). Recently a FeCl₃ system has also been tested for biomass pretreatment (20). Our study shows the importance of taking into account of potential detrimental effect from oxidants or redox-active substances in the enzymatic hydrolysis step for biomass conversion, as well as including such consideration in the overall development/optimization of biomass conversion technologies. Further mechanistic understanding of the interaction between cellulase and oxidized cellulose and biophysical characterization of oxidized cellulose (quantification and localization of oxidized sites) could assist us in finding and engineering cellulases more tolerant of this oxidative inhibition effect.

ACKNOWLEDGEMENTS

We thank Robert L. Starnes and Claus C. Fuglsang from Novozymes for critical reading of the manuscript. This material is based upon work supported by the Department of Energy under Award number DE-FC36-08GO18080. This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of the authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.
REFERENCES


TABLE 1. Inhibition parameter $I_{50}$ (mean ± SD, in mM) of Fe(II) on enzymatic cellulolysis

<table>
<thead>
<tr>
<th>Cellulase mix</th>
<th>CEL7A</th>
<th>CEL6A</th>
<th>CEL7B</th>
<th>CEL5A</th>
<th>CEL3A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avicel</td>
<td>2 ± 1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PASC</td>
<td>14 ± 1</td>
<td>7 ± 2</td>
<td>~7</td>
<td>1.8 ± 0.2</td>
<td>12 ± 4</td>
</tr>
</tbody>
</table>

ND: Not determined. $^a$ On cellobiose hydrolysis.
<table>
<thead>
<tr>
<th>Ion</th>
<th>Charge</th>
<th>Radius, Å</th>
<th>$E^0$, V</th>
<th>Effect on enzymatic cellulolysis$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg(II)</td>
<td>2+</td>
<td>0.72</td>
<td></td>
<td>~20% gain in initial rate, ~20% gain in extended hydrolysis extent</td>
</tr>
<tr>
<td>Ca(II)</td>
<td>2+</td>
<td>1.00</td>
<td></td>
<td>~20% gain in initial rate, ~20% gain in extended hydrolysis extent</td>
</tr>
<tr>
<td>Cr(III)</td>
<td>3+</td>
<td>0.62</td>
<td>-0.407</td>
<td>~40% loss in initial rate, ~40% loss in extended hydrolysis extent</td>
</tr>
<tr>
<td>Mn(II)</td>
<td>2+</td>
<td>0.67</td>
<td></td>
<td>~10% loss in initial rate, no change on extended hydrolysis extent</td>
</tr>
<tr>
<td>Fe(II)</td>
<td>2+</td>
<td>0.61</td>
<td></td>
<td>~70% loss in initial rate, ~70% loss in extended hydrolysis extent</td>
</tr>
<tr>
<td>Fe(III)</td>
<td>3+</td>
<td>0.55</td>
<td>0.771</td>
<td>~90% loss in initial rate, ~90% loss in extended hydrolysis extent</td>
</tr>
<tr>
<td>Co(II)</td>
<td>2+</td>
<td>0.65</td>
<td></td>
<td>~10% loss in initial rate, no change on extended hydrolysis extent</td>
</tr>
<tr>
<td>Ni(II)</td>
<td>2+</td>
<td>0.69</td>
<td></td>
<td>~20% loss in initial rate, ~10% loss in extended hydrolysis extent</td>
</tr>
<tr>
<td>Cu(II)</td>
<td>2+</td>
<td>0.73</td>
<td>0.153</td>
<td>~90% loss in initial rate, ~80% loss in extended hydrolysis extent</td>
</tr>
<tr>
<td>Ru(III)</td>
<td>3+</td>
<td>0.68</td>
<td>0.249</td>
<td>~90% loss in initial rate, ~80% loss in extended hydrolysis extent</td>
</tr>
<tr>
<td>Zn(II)</td>
<td>2+</td>
<td>0.74</td>
<td></td>
<td>~20% loss in initial rate, ~20% loss in extended hydrolysis extent</td>
</tr>
</tbody>
</table>

$^a$The counter anions of these metal ion compounds, $\text{SO}_4^{2-}$ and $\text{Cl}^-$, were inert under the hydrolysis conditions of this study. $^b$ Ionic radii in crystals, for 6-ligand coordination (19). $^c$ Single-electron oxidation potential vs Normal Hydrogen Electrode. Metal ions without $E^0$ entry are stable against either reduction or oxidation under the conditions of this study. $^d$ One-mL scale hydrolysis of 23 g/L Avicel by 0.25 g/L cellulase mix in 50 mM Na-acetate of pH 5, 50°C, 4 days.
TABLE 3. Oxidation potential and inhibitory effect of iron-chelator complexes

<table>
<thead>
<tr>
<th>Complexes</th>
<th>$E^0$, $V^n$</th>
<th>Effect on enzymatic cellulolysis$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe(III)(2,2’BP)Cl$_2$</td>
<td>0.78</td>
<td>~100% loss in initial rate, ~100% loss in extended hydrolysis extent</td>
</tr>
<tr>
<td>Fe(II)(2,2’BP)Cl$_2$</td>
<td></td>
<td>~40% loss in initial rate, ~40% loss in extended hydrolysis extent</td>
</tr>
<tr>
<td>K$_2$Fe(III)(CN)$_6$</td>
<td>0.358</td>
<td>No significant change on initial rate, no change on extended hydrolysis extent</td>
</tr>
<tr>
<td>K$_3$Fe(III)(CN)$_6$</td>
<td></td>
<td>~10% loss in initial rate, ~20% loss in extended hydrolysis extent</td>
</tr>
<tr>
<td>Fe(III)NaEDTA</td>
<td>0.13$^b$</td>
<td>~10% loss in initial rate, ~20% loss in extended hydrolysis extent</td>
</tr>
<tr>
<td>Fe(II)Na$_3$EDTA</td>
<td></td>
<td>~80% loss in initial rate, ~80% loss in extended hydrolysis extent</td>
</tr>
<tr>
<td>Fe(III)-citrate</td>
<td>-0.191$^b$</td>
<td>No significant change on initial rate, no change on extended hydrolysis extent</td>
</tr>
</tbody>
</table>

$^a$ Single-electron oxidation potential (19). $^b$ From ref. (3, 6). Iron-chelator complexes without $E^0$ entry are reduced counterparts of corresponding oxidized complexes ($E^0$ given). $^d$ Hydrolysis of 23 g/L Avicel by 0.25 g/L cellulase mix in 50 mM Na-acetate of pH 5, 50°C, 4 days.
TABLE 4. $I_{50}$ (in mM) of selected inhibitors $^a$

<table>
<thead>
<tr>
<th></th>
<th>FeCl$_3$</th>
<th>Fe(2,2’BP)$_3$Cl$_3$</th>
<th>Fe(2,2’BP)$_3$Cl$_2$</th>
<th>FeNaEDTA</th>
<th>FeNa$_2$EDTA</th>
<th>CuSO$_4$</th>
<th>RuCl$_3$</th>
<th>CrCl$_3$</th>
<th>Quinone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 ± 0.4</td>
<td>0.6 ± 0.1</td>
<td>−20</td>
<td>−10</td>
<td>&lt;10</td>
<td>−0.2</td>
<td>−0.15</td>
<td>&gt;10</td>
<td>−5</td>
</tr>
</tbody>
</table>

$^a$ Tested in 23 g/L Avicel hydrolysis by 0.25 g/L cellulase mix
FIGURE LEGENDS

FIG. 1. Effect of Fe(II) and other ions on enzymatic PCS hydrolysis. Added salt concentrations: 10 mM. Hydrolysis: 43 g/L PCS, 0.25 g/L cellulase mix, 50 mM Na-acetate, pH 5, 50°C. Similar results were observed with cellulase mix.

FIG. 2. Effect of Fe(II) on individual cellulases. (A) CEL7A CBH-I, (B) CEL6A CBH-II, (C) CEL7B EG-I, (D) CEL5A EG-II, (E) CEL3A BG. Inhibitor: 10 mM FeSO₄. Hydrolysis: 2 g/L PASC (for (A)-(D)) or cellobiose (for (E)), 40 (for (A)-(D)) or 1 (for (E)) mg/L cellulase, 50 mM Na-acetate, pH 5, 50°C.

FIG. 3. Correlation between redox potential and cellulolysis-inhibiting effect of selected (A) oxidative metal ions and (B) Fe-ligand complexes (also including p-quinone). Data for E° and initial rate of cellulase mix-catalyzed Avicel hydrolysis were from Tables 2&3 and text. Correlation line: (A) relative rate = -48E° + 29, r² = 0.795; (B) relative rate = -97E° + 88, r² = 0.766.

FIG. 4. Effect of Fe(II) oxidation and Fe(III) chelation on enzymatic cellulolysis. Hydrolysis: 43 g/L PCS, 0.25 g/L cellulase mix, 50 mM Na-acetate of pH 5, 50°C. Concentration of inhibitor, oxidant, and chelator added to the hydrolysis suspension: FeSO₄: 2.5 mM, H₂O₂: 10 mM, desferrioxamine: 10 mM.
Fig. 1

Hydrolysis, %  
Time, day

(○) Cellulases alone
(-) Cellulases plus NaCl, Na₂SO₄, MnCl₂, or MnSO₄
(□) Cellulases plus FeSO₄
(◊) Cellulases plus FeCl₂
Fig. 2

A
(○) CEL7A alone
(□) CEL7A plus Fe(II)

B
(○) CEL6A alone
(□) CEL6A plus Fe(II)

C
(○) CEL7B alone
(□) CEL7B plus Fe(II)

D
(○) CEL5A alone
(□) CEL5A plus Fe(II)

E
(○) CEL3A alone
(□) CEL3A plus Fe(II)
Fig. 4

A
(○) Cellulases alone
(□) Cellulases plus Fe(II) and H$_2$O$_2$
(◊) Cellulases plus Fe(II) and H$_2$O$_2$ and desferrioxamine
(∆) Cellulases plus Fe(II) and H$_2$O$_2$ and desferrioxamine

B
(○) Cellulases alone
(+) Cellulases plus H$_2$O$_2$
(×) Cellulases plus H$_2$O$_2$ and desferrioxamine

Hydrolysis, %
Time, day