Studies on the Mechanism of Alkaline Peroxide Delignification of Agricultural Residues

J. Michael Gould
Northern Regional Research Center,
Agricultural Research Service,
U.S. Department of Agriculture,*
Peoria, Illinois 61604

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Alkaline solutions of hydrogen peroxide partially delignify wheat straw and other lignocellulosic materials, leaving a cellulosic residue that is highly susceptible to enzymatic digestion by cellulase. The delignification reaction is strongly dependent upon the pH of the reaction mixture, with an optimum at pH 11.5–11.8. $\text{H}_2\text{O}_2 \rightleftharpoons \text{H}^+ + \text{HOO}^-$. The data are consistent with a mechanism in which $\text{H}_2\text{O}_2$ decomposition products such as -OH and $\text{O}_2^-$, rather than $\text{H}_2\text{O}_2$ or $\text{HOO}^-$, are the primary lignin oxidizing species. During the course of the delignification reaction, $\text{O}_2$ is evolved from the reaction mixture indicating active $\text{H}_2\text{O}_2$ decomposition. At a given concentration of $\text{H}_2\text{O}_2$, the rate of $\text{O}_2$ evolution is proportional to the amount of lignocellulosic substrate present in the reaction mixture. However, the total amount of $\text{O}_2$ evolved is inversely proportional to the amount of substrate present, indicating that some of the peroxide oxygen becomes incorporated into lignin degradation products. The amount of peroxide oxygen incorporated can range as high as 2 $\text{O}_2$ per lignin $\text{C}_5$ unit, depending upon the initial concentration of lignocellulosic substrate.

INTRODUCTION

The rate and extent to which cellulose in lignocellulosic materials can be enzymatically saccharified is limited by two important factors: 1) the close physical and chemical association between lignin and the cell wall polysaccharides, and 2) the degree of crystallinity within the cellulose polymer itself.\textsuperscript{1} Attempts to enhance the efficiency of enzymatic glucose production from lignocellulosics have involved the use of chemical, physical, or biological pretreatments of the material in an effort to remove lignin and/or decrease cellulose crystallinity.\textsuperscript{2} Most of these pretreatments do result in increased glucose yields, and a few allow essentially quantitative conversion of cellulose into glucose. Unfortunately, the more efficient pretreatments often utilize toxic reagents and/or generate inhibitory side products that interfere with microbial growth during subsequent fermentation reactions.\textsuperscript{3–6}

In nature, lignin is degraded by a variety of different organisms. Although the mechanism of natural delignification remains largely unknown, it now is thought that hydrogen peroxide ($\text{H}_2\text{O}_2$) excreted by the organism plays an important role.\textsuperscript{7–9} Hydrogen peroxide has been widely used as a nondelignifying bleach for high-lignin wood pulps,\textsuperscript{10} even though it is known that under proper conditions $\text{H}_2\text{O}_2$ will react readily with lignin and related phenolics to yield an array of low molecular weight, water-soluble oxidation products.\textsuperscript{11–15} Recently, we demonstrated that dilute, alkaline solutions of $\text{H}_2\text{O}_2$ will solubilize about one-half of the lignin from a variety of lignocellulosic crop residues.\textsuperscript{17} Most of the hemicellulose originally present was also solubilized by this treatment. The insoluble, cellulose-rich fraction that remained was highly susceptible to \textit{Trichoderma reesei} cellulase, with cellulose to glucose conversion efficiencies close to 100% for wheat straw and corn stover. In combined saccharification/fermentation experiments using cellulase and \textit{Saccharomyces cerevisiae}, ethanol yields from these crop residues exceeded 90% of theoretical (based upon original cellulose content), indicating that the lignin degradation products formed during the delignifying reaction were not inhibitory to microbial growth.\textsuperscript{18}

The mechanism by which alkaline peroxide pretreatment enhances enzymatic saccharification appears to involve both a release of lignin from the lignocellulosic matrix and a dramatic increase in the degree of hydration of the cellulose polymer. In this article, we report additional studies on the mechanism of alkaline peroxide action that support this conclusion.

EXPERIMENTAL METHODS

Substrates

Most of the experiments in this study were performed using wheat straw (obtained locally) as a representative lignocellulosic agricultural residue. The straw was ground in a Wiley mill to pass a 2-mm screen and...
then stored in polyethylene containers until use. For some experiments, corn stover (stalks and husks), oak shavings, or \(^1\)C-lignin labelled kenaf served as the substrate. Corn stover and labelled kenaf were prepared in a manner similar to that described above for wheat straw. Lignin in the kenaf was labelled by feeding the plant \(^1\)C-phenylalanine, as described elsewhere.\(^{19}\)

Alkaline Peroxide Pretreatment

Wheat straw, corn stover, and oak shavings were treated with H\(_2\)O\(_2\) by incubating 1 g of the lignocellulosic substrate in 50 mL deionized water containing 1% H\(_2\)O\(_2\). NaOH was added to bring the suspension to pH 11.5, and the mixture was stirred gently at room temperature. In some experiments, reaction conditions were varied as indicated in the figure legends.

When \(^1\)C-lignin kenaf served as substrate, reactions were run on a smaller scale, with 20 mg labelled kenaf in 1.0 mL 1% H\(_2\)O\(_2\), pH 11.5. The amount of lignin solubilized at various times during the treatment was determined by measuring the radioactivity of a 25 \(\mu\)L aliquot of the reaction mixture aqueous phase using liquid scintillation spectrometry. Specific activity of the \(^1\)C-labelled kenaf was 5600 dpm/mg.

The efficiency with which cellulose in the peroxide-treated residues could be hydrolyzed to glucose by *Trichoderma reesei* cellulase was determined as described elsewhere.\(^{17}\)

Oxygen Evolution

The amount of oxygen evolved during the alkaline peroxide reaction with wheat straw was determined manometrically. The maximum oxygen evolution possible was determined empirically by adding catalase to a duplicate reaction mixture not containing straw.

Electron Microscopy

Wheat straw was treated with alkaline peroxide as described above, and then either oven dried (110°C for 24 h) or dehydrated by sequential ethanol washes (20% concentration steps) and critical point dried (CO\(_2\)). Samples were examined in an ISI model SS130 scanning electron microscope.

RESULTS

Changes in Physical Properties

Previous studies have shown that wheat straw treated with alkaline hydrogen peroxide released a maximum of about half of its lignin as water-soluble degradation products.\(^{15}\) We also noted that, as the delignification reaction approached completion, the straw particles disintegrated into small, highly dispersed fibers, and the suspension acquired a more homogeneous, pulplike consistency.\(^{18}\) This loss of physical and morphological integrity is detailed in the scanning electron micrographs shown in Figure 1. The original (untreated) straw particles had essentially completely disintegrated after 18 h in 1% H\(_2\)O\(_2\), pH 11.5, leaving a dispersion of small fibers with a fairly broad range of sizes. Higher magnification views of these fibers revealed a considerable amount of disruption within the fiber bundles.

Disintegration of straw occurred most readily after the H\(_2\)O\(_2\)-dependent lignin degradation reaction (50–60% delignification) was practically complete (6–8 h, 25°C), and appeared to require some degree of mechanical agitation to occur maximally. When the alkaline peroxide pretreatment was performed with swirling provided by a gyro-rotary shaker (200 rpm), for instance, only a small amount of the straw disintegrated in 24 h. When stirring was provided by a magnetic stirring bar, however, much more of the straw disintegrated in 24 h. Not all of the materials tested lost physical and morphological integrity equally. Corn stalks and husks appeared to disintegrate somewhat more readily than straw, whereas oak shavings disintegrated only when the suspension was subjected to strong mechanical agitation (e.g., 60 s in a Waring Blender). Intact straw disintegrated as readily as ground straw, although the fibers tended to be much longer. Intact straw incubated in alkaline peroxide without any stirring at all became flaccid, but it did not disintegrate until mild mechanical agitation (e.g., gentle stirring) was supplied. None of the morphological changes described above were observed in samples incubated under similar conditions at pH 11.5 in the absence of H\(_2\)O\(_2\).

The straw's loss of physical integrity during alkaline peroxide treatment was accompanied by a dramatic increase in the water absorbancy of the cellulose-enriched residue (Table I). Native straw (not treated) absorbed water equivalent to ca. eight times its dry weight. Treatment of the straw at pH 11.5 for 24 h in the absence of H\(_2\)O\(_2\) increased its water absorbancy only slightly. Treatment at pH 11.5 with 1% H\(_2\)O\(_2\), on the other hand, increased the water absorbancy of the residue by 300%. The higher water absorbancy in H\(_2\)O\(_2\)-treated straw reflects an increase in the level of hydration of the straw cellulose, suggesting a significant decrease in the proportion of total cellulose contained in highly crystalline structures. This conclusion is further supported by the ease and rapidity with which the cellulose in H\(_2\)O\(_2\)-treated straw can be enzymatically hydrolyzed.\(^{17}\) Interestingly, the high water absorbancy of peroxide-treated straw was not seriously diminished by oven drying (Table I).

Effect of pH

The extent of the delignification reaction catalyzed by H\(_2\)O\(_2\) was a strong function of the reaction pH. In the absence of H\(_2\)O\(_2\), the maximum amount of lignin that was solubilized in a 24-hr treatment increased...
steadily with increasing pH (Fig. 2). Very little lignin was solubilized below pH 11, whereas as much as 30% of the lignin was solubilized at pH 13. In the presence of 1% H₂O₂, substantially more lignin was solubilized over the entire pH range tested. There was an apparent optimum around pH 11.5, where the degree of delignification approached 60%, but similar levels of delignification were also attained using H₂O₂ at pH >12.5. The pH optimum for H₂O₂-catalyzed delignification became even more apparent when the delignification occurring in the presence of H₂O₂ was corrected for delignification occurring in the absence of H₂O₂ (Fig. 3), with the corrected pH profile exhibiting a relatively sharp peak around pH 11.5. Similar results were obtained with both kenaf (Fig. 2) and wheat straw.

Table 1. Water absorption by alkaline-peroxide-treated wheat straw.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>H₂O absorption (g H₂O/g solid)</th>
</tr>
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<tbody>
<tr>
<td>Untreated</td>
<td>7.9</td>
</tr>
<tr>
<td>pH 11.5</td>
<td>10.3</td>
</tr>
<tr>
<td>pH 11.5, 1% H₂O₂</td>
<td>23.7</td>
</tr>
<tr>
<td>pH 11.5, 1% H₂O₂ (oven dried)</td>
<td>19.7</td>
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</table>

* Treatments were performed for 24 hr using 1 g straw in 50 mL final volume.

* Water absorption was measured by soaking the residue for 20 min in a large excess of deionized water, allowing the excess water to drain through a fine mesh screen, and then weighing the saturated residue before and after drying at 110°C for 24 h.

* After the alkaline peroxide treatment the insoluble residue was dried at 110°C for 24 h before determining water absorption.

Figure 2. Effect of reaction pH on the extent of lignin solubilization from 14C-lignin kenaf. Samples were treated at the indicated pH in the (●) presence or (○) absence of 1% H₂O₂ for 24 h at 25°C.
Figure 3. Efficiency of the H₂O₂-dependent delignification of ¹⁴C-lignin kenaf as a function of the initial pH of the reaction mixture. The data shown here were obtained from the data in Figure 2 by subtracting the amount of lignin solubilized in the absence of H₂O₂ from the amount of lignin solubilized in the presence of H₂O₂ at each reaction pH tested.

Not surprisingly, the effects of pH on delignification efficiency closely paralleled the effects of treatment pH on the efficiency with which cellulose in H₂O₂-treated residues could be saccharified enzymatically. More than 90% of the cellulose present in wheat straw could be converted to glucose when the straw was treated with H₂O₂ at pH 11.5, compared to only about 30% conversion for straw treated at pH 11.5 in the absence of H₂O₂. However, glucose yields from straw treated at pH 7–10 or pH 12.5–13 were not significantly enhanced by the presence of H₂O₂ during the treatment.

The saccharification efficiencies for straw samples that had been pretreated for different periods of time with H₂O₂ at pH 11.5 were strongly related to the extent of delignification of the sample, with maximal saccharification efficiency attained when 50% or more of the straw lignin had been solubilized (Fig. 4). This relationship was also observed for samples treated at pH 10–11.5, but did not hold for samples treated with H₂O₂ at pH 12.5–13. Under these conditions, only about 70% of the cellulose was saccharified by cellulase, even though more than 50% of the straw lignin had been solubilized.

Oxygen Consumption and Evolution

When wheat straw was incubated in an alkaline solution of hydrogen peroxide, O₂ gas was evolved from the reaction mixture (Fig. 5) as a result of decomposition of some of the H₂O₂. Both the rate and extent of O₂ evolution were dependent upon the amount of lignocellulosic substrate added to the reaction mixture. Increasing levels of substrate resulted in faster rates of O₂ evolution, with an initial rate of about 0.5 mmol O₂ evolved/h/g straw added (Fig. 6).

Assuming the overall reaction for the decomposition of hydrogen peroxide to be

\[ 2H₂O₂ → 2H₂O + O₂ \]  (1)

it is possible to calculate the maximum amount of O₂ that could be generated from a given amount of H₂O₂. Alternatively, the enzyme catalase, which catalyzes reaction (1), can be added to a duplicate reaction
Figure 6. Effect of straw concentration on the initial rate of O$_2$ evolution during alkaline peroxide treatment. Reaction conditions were as described in Figure 5 except the straw concentration was varied as indicated.

mixture (minus straw), and the amount of O$_2$ evolved can be measured directly. It is clear from the data in Figure 5 that the amount of O$_2$ evolved in the presence of wheat straw was always less than the maximum, with increasing levels of substrate resulting in decreasing amounts of evolved O$_2$. The difference between the amount of O$_2$ actually evolved and the maximum possible O$_2$ yield (determined from the initial H$_2$O$_2$ content) was assumed to represent the amount of O$_2$ consumed in the delignification reaction and incorporated into lignin degradation products. As shown in Figure 7, the amount of O$_2$ consumed increased with increasing levels of wheat straw up to about 6% (w/v) straw (3 g/50 mL). At higher straw concentrations, the amount of O$_2$ consumed in the delignification reaction remained essentially constant. In other words, the amount of peroxide oxygen ultimately incorporated into lignin degradation products (at a given H$_2$O$_2$ concentration)

decreased as the amount of straw present in the reaction mixture was increased. Assuming a wheat straw lignin content of 15% (dry weight basis) and a molecular weight of 150 for a typical C$_9$ lignin monomeric unit, it was possible to calculate the amount of oxygen incorporated into lignin products as a function of the straw concentration during the reaction with H$_2$O$_2$. At low straw concentrations, the amount of O$_2$ incorporated/C$_9$ unit approached 2; but as the straw concentration was increased, the level of incorporation decreased rapidly (Fig. 8). At higher straw concentrations (>6%), significant delignification did not occur, and the resultant increases in cellulase digestibility were not attained. This is most likely the result of H$_2$O$_2$ depletion in the reaction mixture (to form O$_2$) via rapid, non-delignifying reactions (see below).

DISCUSSION

Alkaline hydrogen peroxide is widely used as a bleach in the production of pulp and paper. In this application, which is generally considered to be non-delignifying, the peroxide is usually stabilized against decomposition by sodium silicate or MgSO$_4$. In the absence of stabilizers H$_2$O$_2$ reacts readily with a variety of lignin model compounds and lignocellulosic substrates, producing low molecular weight degradation products. Gellerstedt et al. have shown that it is the intermediate breakdown products formed during the decomposition of H$_2$O$_2$ that are the primary reacting species in alkaline peroxide reactions with lignin model compounds.

In order to understand the mechanism of the alkaline peroxide delignification reaction, therefore, it is necessary to examine more fully the reactions involved in H$_2$O$_2$ decomposition. At alkaline pH, H$_2$O$_2$ dissociates to form the hydroperoxy anion (HOO$^-$)

$$H_2O_2 \rightleftharpoons HOO^- + H^+$$  (2)
with a pKa of 11.6. The hydroperoxy anion can in turn react with undissociated H₂O₂ to form highly reactive hydroxyl radicals (·OH) and superoxide (O₂⁻) in the following reaction:

\[ \text{H}_2\text{O}_2 + \text{HOO}^- \rightarrow \cdot\text{OH} + \text{O}_2^- + \text{H}_2\text{O} \]  \hspace{1cm} (3)

In the absence of other reactants, hydroxyl and superoxide radicals will react with each other to form O₂ and water:

\[ \cdot\text{OH} + \text{O}_2^- + \text{H}^+ \rightarrow \text{O}_2 + \text{H}_2\text{O} \]  \hspace{1cm} (4)

so that the overall reaction, eqs. (3) and (4), becomes

\[ \text{H}_2\text{O}_2 + \text{HOO}^- + \text{H}^+ \rightarrow \text{O}_2 + 2\text{H}_2\text{O} \]  \hspace{1cm} (5)

From eq. (5) it is clear that the maximum amount of O₂ that can be evolved from an alkaline solution of H₂O₂ equals one-half the molar amount of H₂O₂ originally present [see also reaction (1)]. If other compounds are present that can react with ·OH and/or O₂⁻, in competition with reaction (4), the observed yield of O₂ will be reduced below the theoretical maximum.

When H₂O₂ decomposes under alkaline conditions in the presence of a lignin-containing substrate, less than the theoretical amount of O₂ is evolved (Fig. 6), indicating that at least some of the reactive intermediates formed in the decomposition reaction have been incorporated into oxidized lignin products. The fact that the extent of delignification is maximal at about pH 11.5 suggests rather strongly that these intermediates are generated at least in part via reaction (3), since this reaction is known to be strongly pH dependent,[11] with an optimum at pH 11.6. The decrease in delignification (and saccharification) efficiency for samples treated at pH > 11.5 would seem to eliminate the possibility of significant direct oxidation of lignin by HOO⁻, because the concentration of HOO⁻ in the reaction mixture would actually be increased at pH > 11.5.

Although a mechanism involving reaction (3) could explain the apparent pH dependence of the alkaline peroxide delignification reaction, it does not explain the observation that the rate of O₂ evolution is strongly dependent upon the amount of straw present in the reaction mixture (Fig. 5). This dependence suggests the presence of another H₂O₂ decomposition pathway, such as a Fenton-type reaction \([M = \text{metal ion (e.g., Fe}^{2+}\text{)}]):

\[ \text{H}_2\text{O}_2 + M \rightarrow M^+ + \cdot\text{OH} + \text{OH}^- \]  \hspace{1cm} (6)

\[ \text{H}_2\text{O}_2 + M^+ + 2\text{OH}^- \rightarrow M + \text{O}_2^- + 2\text{H}_2\text{O} \]  \hspace{1cm} (7)

\[ \text{(net) } 2\text{H}_2\text{O}_2 + \text{OH}^- \overset{(M/M^+)}{\rightarrow} \cdot\text{OH} \]

\[ + \text{O}_2^- + 2\text{H}_2\text{O} \]  \hspace{1cm} (8)

In the absence of reactants for ·OH or O₂⁻, these radicals again can react with each other to form O₂ and H₂O [reaction (4)], giving an overall O₂ yield of 0.5 mol O₂/mol H₂O₂. As stated above, the presence of compounds that react with ·OH and/or O₂⁻ will reduce the yield of O₂ significantly.

Taken together, the two pathways for H₂O₂ decomposition outlined above [reactions (3) and (6)-(8)], operating simultaneously, could explain the data presented in this study. During alkaline peroxide treatment of straw, the rate of generation of reactive radicals from H₂O₂ via reaction (3) will be determined mainly by the pH of the reaction medium, and should be largely independent of the straw concentration. Since the extent of delignification and the efficiency of subsequent enzymatic saccharification of the treated straw are also determined largely by the reaction pH (Figs. 2, 3, and 5; see also ref. 17), it seems reasonable to conclude that reaction (3) represented the primary pathway leading to the changes in straw structure and composition characteristic of the alkaline peroxide treatment.

Apparently superimposed upon the decomposition of H₂O₂ via reaction (3), however, is a second H₂O₂ decomposition pathway. This pathway seems to be catalyzed by some component that is introduced into the reaction mixture along with the straw, because the rate of O₂ evolution from the reaction mixture is directly proportional to the amount of straw added (Fig. 5). Furthermore, removal of heavy metal contaminants from the wheat straw (for example, by acid prewash, by addition of chelators, or by alkaline precipitation) essentially eliminated O₂ evolution from the H₂O₂ reaction mixture, even at very high straw concentrations [6-10% (w/v)]. At high straw concentrations, removal of heavy metals also restored delignification efficiency and increased saccharification efficiency to levels comparable to those seen for straw treated with H₂O₂ at low solids concentrations (G. Fanta and J. M. Gould, unpublished observations). The catalyzed H₂O₂ decomposition pathway probably generates reactive intermediates [e.g., reactions (6)-(8)], but the major end product seems to be O₂ gas rather than oxidized lignin degradation products (see Fig. 8). Thus, when the level of straw in the reaction mixture is low, the rate of H₂O₂ decomposition to form O₂ gas is low, and the incorporation of oxygen into lignin is more efficient. At higher straw concentrations the rate of H₂O₂ decomposition to form O₂ gas is increased drastically as the catalyzed decomposition reaction rapidly depletes H₂O₂ from the reaction mixture, thereby inhibiting the incorporation of oxygen into lignin (see Figs. 5-8).

It is clear that lignin is probably the major site of chemical attack in the alkaline peroxide reaction. The observed changes in the physical and morphological properties of the cellulose fibers suggest that at least some portion of the glucose units may also have been modified during the treatment. If true, the percentage of total glucose modified must be quite small (<5%), since ca. 95% or more of the cellulose present in the
insoluble residue after alkaline peroxide treatment is released as glucose upon treatment with cellulase (Fig. 4, see also ref. 17). Modification of only a few percent of the glucose units in cellulose would probably be sufficient to disrupt the hydrogen bonding pattern between chains enough to maintain a highly open structure, which could not reform crystalline regions even after oven drying.

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References