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Improvement of saccharide yield from wood by simultaneous enzymatic delignification and saccharification using a ligninolytic enzyme and cellulase

Short title:

Simultaneous enzymic delignification/saccharification

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Abstract

White-rot fungi are thought to hold promise for development of a delignification pretreatment process for wood biorefinery that is less energy-consuming than current processes. However, the reaction must take place over weeks and consumes non-neglectable amounts of saccharides. To establish a biological process for wood biorefinery would first require establishment of an enzymatic approach to delignification. Such an approach has the potential to lower costs and reduce saccharide loss. Here, we attempted enzymatic delignification reactions using manganese peroxidases (MnP), a lignin-degrading enzyme, under several reaction conditions. The delignification rate from beech wood meal (particle size < 45 μm) of up to 11.0% in 48 h was reached in a MnP reaction supplemented with multiple co-oxidants, glucose, glucose oxidase (GOD) and commercial cellulase. An additional 48-h reaction using fresh MnP/co-oxidants increased the delignification rate to 14.2%. Simultaneous enzymatic delignification and saccharification, which occurs without a need for glucose supplementation, successfully improved the glucose yield to 160% of the reaction without MnP. Development of a more accurate imitation of the mechanisms of delignification that occurs in white-rot fungi has the potential to improve the monosaccharide yield resulting from simultaneous delignification and saccharification.

INTRODUCTION

Wood is the most renewable and sustainable material on earth, and is mostly used as a building material and in paper-pulp manufacturing. Recently, the possibility of using woody materials as substrates in the production of biofuels, platform chemicals, and a wide range of material precursors has become a subject of intense study (1). The primary components of woody materials, namely cellulose, hemicellulose, and lignin, are closely associated, and covalent cross-linkage is thought to occur between lignin and these cellulosic polysaccharides. Lignin constitutes 20-35% of woody plant cell walls, and cellulose fibers are covered by lignin, making the plant cell wall highly resistant to microbial and chemical degradation. Thus, lignin degradation is a key step in carbon recycling in land ecosystems as well as for industrial cellulose use, such as in bio-ethanol production. However, delignification by physico-chemical treatments is not an eco-friendly process, as it requires large amounts of energy and chemical reagents.

Wood-rot basidiomycetes fungi, particularly white-rot fungi, which are primarily responsible for initiating lignin degradation, play an important role in carbon recycling in land ecosystems. Ligninolytic white-rot fungi secrete several nonspecific oxidoreductases, such as manganese peroxidase (MnP), lignin peroxidase (LiP), versatile peroxidase (VP), and laccase, which play critical roles in lignin biodegradation (2). Generally, the growth rates of white-rot fungi are very low compared with those of other microbes such as bacteria. White rot fungi take several weeks to months to delignify lignocellulosic materials and consume non-negligible amounts of sugars in the process (3, 4). Development of an in vitro method for delignification based on ligninolytic enzymes might provide a means to delignify substrates faster than is done by live fungi and with reduced sugar and energy consumption. However, development of such a system is made difficult by

our incomplete understanding of the mechanisms underlying delignification by white-rot fungi. Delignification is a very complex reaction involving ligninolytic enzymes, various accessory enzymes, and chemicals (2, 5). Although there have been a few studies of enzymatic delignification (e.g. (6)), enzymatic delignification has not yet reproduced the efficiency of lignin degradation by live fungi, which results in a dramatic change in the lignin content of wood materials. In this study, we attempted to determine what factors positively affect enzymatic delignification by carrying out the reaction under a variety of conditions. Moreover, we also investigated the impact on delignification rates of simultaneous "enzymatic" delignification and saccharification treatment, using manganese peroxidase and commercial cellulase instead of live fungus generally used for biological delignification.

MATERIALS and METHODS

Strains and MnP production. *Phanerochaete sordida* YK-624 *mnp4*-overexpression transformant BM-65 (7) were used in this study. These fungal strains were maintained on potato dextrose agar (PDA). For MnP production, strain BM-65 was cultured in nitrogen-limited liquid medium containing 1.0% D-glucose, 1.2 mM ammonium tartrate, and 20 mM 2,2-dimethyl succinate (8). A quarter of a PDA plate culture of the strain (9 cm in diameter) was homogenized and put into 200 ml liquid medium in a 500-ml Erlenmeyer flask, and incubated for 3 days at 150 rpm and 30 °C. Then, 7.5 mM veratryl alcohol was added into the culture, and sealed with a rubber cap equipped with two glass tubes. The culture was subsequently incubated for an additional 4 days, with a daily oxygen purge of the flask headspace. The culture fluid was then separated from the mycelia by filtration, and the filtrate obtained was dialyzed overnight against a sodium phosphate buffer

57 solution (50 mM; pH 6.0). Next, the dialysate was subjected to anion-exchange chromatography
58 (DEAE-Sepharose Fast Flow; GE Healthcare) as described in a previous reported (9). Fractions containing
59 MnP activity were combined and dialyzed against water. The partially purified MnP solution was
60 concentrated by ultrafiltration (10-kDa cutoff) and used for subsequent experiments.

61 We used oxidation of 2,6-dimethoxyphenol (DMP), as can be monitored by spectrophotometry at
62 470 nm, as in indicator of MnP activity. The reaction mixtures contained 1 mM DMP, 1 mM $MnSO_4$, and 0.2
63 mM H_2O_2 in 50 mM sodium malonate (pH 4.5). One katal (kat) of MnP activity was defined as the amount of
64 enzyme able to catalyze production of 1 mol per second of the quinone dimer (ε_{470} = 49.6 $mM^{-1}cm^{-1}$) from
65 DMP.

66 **Enzymatic lignin degradation.** Three different sizes of extractives-free lignocellulosic materials (beech
67 wood, cedar wood, and bamboo): 30-80 mesh (180-250 μm; MW250), 100-300 mesh (45-150 μm; MW150)
68 and less than 300 mesh (~45 μm; MW45) were prepared from sawdust by sieving after cutter milling (Wonder
69 Crusher WC-3; Osaka Chemical Co., Ltd.). The air-dried lignocellulosic materials (particle size < 150 μm)
70 were milled without any solvent for 5 h in a cycle consisting of 5 min of milling followed by a 5-min rest,
71 using a planetary ball mill (Pulverisette 6; Fritsch Japan Co., Ltd., Japan) to create ball milled woods (BMWs,
72 13.2 ± 7.8 μm). For batch enzymatic reactions (6 ml), each milled lignocellulosic was suspended at a
73 concentration of 75 mg/ml in 50 mM sodium malonate (pH 4.5) containing $MnSO_4$ (0.5 mM), surfactant
74 (Tween 20 or 80; 0.1%), and MnP solution (80 nkat/ml). The reactions were started by adding glucose oxidase
75 (GOD, 1.25 U/ml; Wako, Japan) and glucose (25 mM), and/or cellulase (20 or 60 FPU; cellulase “Onozuka”
76 RS, Yakult Pharmaceutical Industry Co., Ltd.). GOD and glucose were used for the purpose of supplying

77 hydrogen peroxide for long time at relative lower concentration to the reaction rather than adding hydrogen
78 peroxide directly. The reactions were incubated at 30 °C with shaking at 150 rpm for 24 or 48 h. Negative
79 control reactions without MnP were incubated under the same conditions. 1-Hydroxybenzotriazole (3 mM;
80 HBT), linoleic acid (3 mM; LA), and/or reduced glutathione (3 mM; GSH), were added to indicated reaction
81 mixtures. These three chemicals and Tween 80 were used as co-oxidants that work as high redox radical
82 mediators than Mn-malonate complex (see Discussion section). When reactions were complete, the
83 enzymatically delignified milled lignocellulosic material was recovered by filtration and then washed with
84 100 ml of ultra-pure water. After measuring the dry weight of residual milled lignocellulosic material, the
85 residual lignin content of the dried recovered milled lignocellulosic material was evaluated by the Klason
86 method (Klason lignin (acid-insoluble lignin, KL) and acid-soluble lignin (ASL)), as previously described (7).
87 Total lignin (TL) was expressed as the sum of ASL and KL.
88 For multi-step reactions, after the enzymatic reactions (48-h reaction containing 3 mM HBT, 3 mM
89 LA, 3 mM GST, 0.1% Tween 80, with or without cellulase), beech wood meal was recovered by
90 centrifugation (4,000 × *g* for 10 min), then washed with 50 mM malonate (pH 4.5). The enzymes and
91 chemicals were added to a suspension of recovered wood meal to achieve the same conditions as the first
92 reaction, except for the reacted wood meal. This procedure was repeated every 48 h.
93 **DFRC and alkaline nitrobenzene oxidation analysis.** Derivatization followed by reductive cleavage
94 (DFRC) analysis was performed following the method described in a previous report (10). Dried wood sample
95 (50 mg) in an AcBr stock solution (acetyl bromide/acetic acid 8:92 v/v, 15 mL) were stirred at 50 °C for 2 h.
96 After evaporation, the residue obtained and 50 mg zinc powder were suspended in dioxane/acetic acid/water

(5:4:1, 15 mL) solution. The suspension was stirred under room temperature (25 ºC) for 40 min. Following filtration to remove zinc powder, the solution was acidified to pH 3 by 3% HCl solution. Next, the solution was extracted with dichloromethane and acetylated by anhydrous acetic acid and pyridine for gas chromatography (GC) analysis. The degraded monomers (sinapyl alcohol (Salc) and coniferyl alcohol (Calc)) from wood were quantitatively determined by GC (Hewlett-Packard 5980, Atlanta, GA) using a CP-Sil 8CB column (GL Science, 0.25 mm × 25 m × 0.12 μm film thickness) and a flame ionization detector with He as the carrier gas (1.0 mL/min). GC conditions: initial column temperature, 160 °C, hold for 1 min, ramp at 10 °C/min to 300 °C; injector, 220 °C; FID detector, 300 °C.

Alkaline nitrobenzene oxidation (ANBO) analysis was following the method described in previous report (11). The dried wood sample (50 mg) was autoclaved at 170 ºC for 2.5 h with 4 mL of 2 M NaOH and 0.24 mL of nitrobenzene. The resulting solution was washed with diethyl ether, then the aqueous fraction was acidified with 1 M HCl to pH 2-3. Next, the aqueous fraction was extracted with diethyl ether and the diethyl ether fraction was dried by evaporation. The extract was then derivatized by *N,O*-bis(trimethylsilyl)acetamide, followed by GC analysis to quantify S-type (syringaldehyde and syringic acid) and V-type (vanillin and vanillic acid) monomers. GC conditions: initial column temperature, 140 °C, hold for 1 min, ramp at 10 °C/min to 300 °C; injector, 250 °C; FID detector, 300 °C.

Statistical analysis. Data are presented as the average of three replicates of each experiment. Two-way analysis of variance (ANOVA) and the Student's t-test were used to assess significance of differences. A p value <0.05 was considered indicative of statistical significance.

RESULTS

We first sought to evaluate the degradation of lignin, and in particular, KL, in beech wood meal (particle size: less than 150 μm) treated with MnP partially purified from the culture fluid of strain *P. sordida* BM-65. To do this, milled wood was directly added to a MnP reaction mixture along with co-oxidants. When Tween 80 containing unsaturated fatty acid residue was added, a slight but significant level of lignin degradation was observed in our reaction system (Fig. 1A). A control without MnP showed no lignin degradation, although 2.5% of wood weight was lost, which might due to the decomposition of polysaccharides by hydrogen peroxide or other reactive oxygen species in the reaction mixture. Moreover, we observed less than 0.5% degradation of TL and no degradation of KL in a reaction done in the presence of Tween 20. In the enzymatic delignified wood meal reacted with Tween 80 as the co-oxidant, KL degradation was clearly improved as compared with the sample reacted with Tween 20, while degradation of ASL was not affected (Fig. 1A). In addition, the TL and KL contents of the residual wood remaining after reaction in the sample supplemented with Tween 80 were also clearly lower than TL and KL content in the reacted sample supplemented with Tween 20 (Fig. 1B). There was no significant difference in the degree of enzymatic delignification at Tween 80 concentrations in the range 0.1-0.5% (0.76-3.8 mM). Using smaller wood meal particles as a substrate led to better lignin degradation (Fig. 1C). If the wood particle size was lower than 45 μm (MW45), 8% TL degradation was reached in a 24-h reaction period. Figure 1D shows time courses of enzymatic degradation of TL, KL and ASL in MW45. Most delignification in the reaction supplemented with Tween 80 was completed during the first 24 h of incubation, and a slight delignification progressed during the subsequent 24 h. During the reaction, the degree of ASL degradation was nearly constant and it looks like the

progression of TL degradation was dependent on KL degradation.

In the next experiment, multiple co-oxidants were added to the MnP-delignification reactions along with MW150. Figure 2A shows that delignification was slightly improved following addition of each co-oxidant. TL and KL contents in residual wood meal also tended to gone down following addition of each co-oxidant (Fig. 2B). Furthermore, a reaction carried out in the presence of four types of co-oxidants (Tween 80, 1-HBT, LA, and reduced GSH) showed a significantly higher rate of delignification (6.9 ± 0.5%) as compared to a reaction carried out in the presence of only Tween 80 (4.0 ± 0.1%).

We next investigated the effect of wood particle size, cellulase addition, removal of water-soluble fraction and tree species on enzymatic delignification in the presence of the set of four co-oxidants noted above. As for Tween 80 alone, use of smaller particle wood meal led to better enzymatic lignin degradation in the presence of the four co-oxidants (Fig. 3A). In the presence of the four co-oxidants, the rate of delignification was improved by about 1.9% in MW150 and 0.8% in MW45 as compared a Tween 80-only control (Fig. 1C). The influence of cellulase addition on delignification by the MnP/co-oxidants reaction (MW45) are shown in Fig. 3B. Cellulase supplementation of the MnP/co-oxidants reaction improved the efficiency of delignification by 26% as compared with a reaction without cellulase (8.8% vs. 10.5%). When cellulase was added to the first reaction and the second reaction following wash was performed without cellulase, the delignification rate achieved was 14.2%, an improvement of 1.36 times over the 2-step reaction without cellulase addition. In addition, the second reaction after the first delignification reaction with cellulase showed 3.2% additional delignification, while the second reaction after the reaction without cellulase was showed only 1.7% additional delignification. These results suggest that the lignin surface area is an important

157 factor for the MnP/co-oxidants delignification reaction. Repeated enzymatic delignification with “reaction
158 then wash” cycles advanced lignin degradation as shown in Fig. 3C. The rate of delignification was increased
159 to 13.6% on the third reaction. However, lignin degradation was not improved in subsequent fourth and fifth
160 reactions. Therefore, we performed DFRC and ANBO analysis to estimate the change of lignin structure of
161 the delignified wood after first and third reactions and raw BMW (Table 1). In DFRC analysis, recovery of
162 monomers (Salc and Calc) from delignified wood after the first reaction was significantly decreased, and
163 recovery of these monomers further decreased after the third reaction. The ratio of Salc vs. Calc (S/G ratio)
164 clearly dropped, by almost 50%, after the first enzymatic delignification. However, there was no difference
165 between the S/G ratios of first and third reaction samples. These results suggest that the MnP/co-oxidants
166 reaction degrade syringil type β-O-4 structure prior to guaiacyl type in the first reaction, then both are
167 degraded during subsequent reaction steps. Although the ratios of recovered S-type and V-type monomers in
168 ANBO analysis tended to increase slightly in samples after enzymatic delignification, no significant
169 differences were observed between the reacted and raw wood samples. Recovery of monomers from the
170 reacted woods was around 80% of raw wood. These values, in particular the values for S-type monomers, are
171 clearly higher than monomer recovery from DFRC analysis. Figure 3D shows the results of enzymatic
172 delignification of cedar and bamboo wood meal in addition to beech wood meal. The highest rate of lignin
173 degradation was observed in beech, followed by bamboo and then cedar.

174 Finally, we attempted simultaneous enzymatic delignification and saccharification of beech wood
175 meal (Fig. 4). This reaction contained MnP, Mn(II), co-oxidant(s), and cellulase, without glucose
176 supplementation. After 48 h, the reaction supplemented 60 FPU cellulase showed 1.5 times higher lignin

177 degradation than the reaction with 20 FPU cellulase, regardless of the number of co-oxidants added. After
178 comparing single co-oxidant supplementation with 4 co-oxidants supplementation, we found that the 4
179 co-oxidants supplemented reaction showed slightly but significantly higher lignin degradation (118%) at 60
180 FPU cellulase addition, as well as a tendency towards higher lignin degradation (P=0.08) at 20 FPU cellulase
181 addition. Glucose recovery in the reactions with 20 or 60 FPU cellulase were 1.4 or 1.6 times higher than
182 control (without MnP), respectively, regardless of the number of co-oxidants. Independent of cellulase activity,
183 the reaction that included four co-oxidants tended to have a relatively higher glucose recovery (110%) as
184 compared with the reaction with Tween 80 only (p=0.06 at 20 FPU and p=0.09 at 60 FPU). These results
185 indicate that better delignification leads to better saccharification, and that a higher rate of saccharification
186 rate leads to a higher rate of delignification. It seems that enzymatic delignification and saccharification
187 mutually complement each other.
188
189 **DISCUSSION**
190 White-rot fungi are able to delignify lignocellulosic materials; for example, *P. sordida,* which
191 mainly secrets MnP as a ligninolytic enzyme, eliminates 40-50% of lignin from cutter milled beech wood
192 meal after several weeks of cultivation (7). It has already been shown that chemicals known as co-oxidants
193 play an important role in oxidizing non-phenolic structures including lignin (12). Unsaturated fatty acids play
194 an important role in the oxidation of non-phenolic lignin structures during the delignification reaction carried
195 out by *Ceriporiopsis subvermispora*, which secretes MnP as its main ligninolytic enzyme (13). Nevertheless,
196 addition of co-oxidants such as GSH and Tween 80 to MnP reactions leads to higher levels of delignification

197 of BMW (14-16). It has been proposed that radicals of these co-oxidants attack non-phenolic lignin structures
198 to form benzyl radicals, then β-aryl ether and C_α-C_β bonds are cleaved or corresponding α-keto structures are
199 formed (12). As a result, MnP improves lignin depolymerization activity in the presence of such co-oxidants.
200 In addition to MnP, several other compounds that function as redox mediators in the laccase reaction are
201 known, including HBT and 3-hydroxyanthranilic acid (17). These findings clearly indicate that these
202 co-oxidants improve catalysis by MnP of the delignification reaction. However, BMWs were used in those
203 previous reports. Ball milling depolymerizes lignocellulose and increases phenolic β-O-4 content (18), and
204 this is associated with increased MnP reactivity. There had been no report that quantitatively proves
205 MnP/co-oxidants degrade lignin in native wood (non-BMW). Additionally, as rates of delignification
206 equivalent to the high rates that can be achieved by white-rot fungi have not yet been reproduced *in vitro*, we
207 propose that there are unknown factors affectable to the delignification reaction present in the fungi additional
208 to co-oxidants and hydrogen peroxide production system. Therefore, we first attempted to the enzymatic
209 delignification reaction under several conditions to identify factors that affect the reaction.

210 Consistent with previous reports, supplementation of co-oxidants improved the efficiency of MnP
211 enzymatic delignification, and as expected, increasing the lignin surface area via wood fine grinding and
212 enzymatic saccharification also improved delignification (Fig. 1-3). In addition, supplementation of the
213 reaction with multiple co-oxidants led to a slight but clear improvement in the rate of lignin degradation.
214 Repeated “reaction then wash” cycles were able to extend the rate of lignin degradation; however, the effects
215 of repeating the reaction were limited (Fig. 3C). This result suggests that presence of water-soluble products is
216 not the main limiting factor for enzymatic delignification. From these results, it was clarified that

MnP/co-oxidants reaction system degrade lignin containing in both non-BMW and BMW by adding various co-oxidants in combination without any chemical treatment. This is first report to demonstrate clear enzymatic delignification of native lignin by Klason method which is an intuitive method for the determination of lignin content.

Characterization of the structure of residual lignin in delignified wood after the first and third reaction cycle was performed by DFRC, which produces lignin monomers (Salc and Calc) from β-O-4 structures (19) and by ANBO, which produces S-type and V-type monomers from uncondensed structure (11). The recovery of monomers from syringyl β-O-4 structures by DFRC was significantly decreased (55-73%) in the MnP/co-oxidants reaction sample, while recovery of S-type monomers from uncondensed structures decreased only 15-18%. On the other hand, such large differences were not observed for Calc and V-type monomer recovery. Calc and V-type monomers from the third reaction sample were recovered at levels 70% and 77%, respectively, as compared with levels obtained with control wood. Generally, it is thought that β-O-4 linkages account for 45-50% of the phenylpropane unit bonds in lignin (20), and that MnP/co-oxidant reactions mainly attack Cα-position to cleave β-O-4 linkages (12). Phenolic structures produced during lignin degradation are re-oxidized by ligninolytic enzymes and repolymerized to form uncondensed structures such as β-O-4 and more stable condensation linkages. In the case of oxidation polymerization of monolignols by horse radish peroxidase (21), sinapyl alcohol tends to form β-β and β-1 structures in addition to β-O-4 structures, and these structures can be converted to S-type monomers by ANBO (21, 22). Coniferyl alcohol is polymerized more easily than sinapyl alcohol and forms β-5 and 5-5 condensed linkages, which do not form V-type monomers in the presence of ANBO (23). Based on these observations, it is estimated that termination

237 of the MnP/co-oxidants reaction was caused by repolymerization of lignin-degrading products. Moreover, as a
238 result of repolymerization of lignin-degrading fragments, syringil type un-condensed structures other than
239 β-O-4 and guaiacyl type condensed and uncondensed structures might have remained in residual lignin. Thus,
240 we presumed that a decrease in the yield of V-type monomer from ANBO was occurred.

241 We accomplished 8.8% lignin degradation from beech MW45 and 10.4% from BMW in single
242 reactions (Fig. 3A). Furthermore, combining MnP/co-oxidants with cellulase significantly improved
243 delignification of MW45, and application of a multiple cycle reaction further improved the efficiency of
244 enzymatic delignification, to over 13% (Fig. 3B and C). Thus, we next attempted simultaneous “enzymatic”
245 delignification and saccharification. Typically, biological delignification is usually done with live white-rot
246 fungus, we employed the enzyme-only reaction without using any live fungal treatment in this experiment.
247 The reaction contained MnP, co-oxidants and cellulase, as for previous reactions, but glucose was excluded.
248 Although the rate of delignification was lower than that observed for the reaction supplemented with glucose
249 at the start, the results suggest that delignification and saccharification mutually complemented on another. If
250 simultaneous MnP/co-oxidants reaction significantly improved enzyme saccharification, glucose yields could
251 increase 160% as compared to the yield from reaction without delignification, regardless of the dose of
252 cellulase. The simultaneous delignification and saccharification treatment of only enzymatic reaction was
253 insufficient at present, but was able to improve the sugar yields. It shows the potential for development of
254 enzymatic process for monosaccharide production from lignocellulosic materials without excessive sugar
255 consumption and fermentation inhibitors production.

256 In the present study, it is shown that number of co-oxidant types is an important factor for MnP

enzymatic delignification reaction, besides lignin surface area which can be increased physical and enzymatic treatment. However, amount of delignification was not enough for analysis detail of lignin chemical structure by using nuclear magnetic resonance spectrum. The cause of this is possibly re-polymerization of lignin degrading fragments to form condensed structures which are stable for MnP/co-oxidants reaction system. It is expected that white-rot fungi possess the mechanism to prevent re-polymerization of lignin-degrading fragments in addition of co-oxidants production system to degrade lignin effectively. By adding a means to prevent repolymerization of lignin-degrading fragments to the enzymatic reaction system, the highly efficient lignin-degrading mechanism of white-rot fungi might be more accurately approximated. In addition, if the reaction efficiency can be improved, then it is expected that the simultaneous enzymatic delignification and saccharification reaction will be able to result in higher glucose yields.

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Figure legends

Fig. 1. Effect of Tween 80 supplementation for MnP catalyzing delignification reaction. The reactions contained various concentration of Tween 20 (T20) or 80 (T80) in addition to MnP, GOD, glucose, $MnSO_4$ and beech wood meal. A) and B) show lignin degradation for each reaction and lignin contents in residual wood after a 24 h enzymatic reaction with MW150 as a substrate. C) shows 24 h lignin degradation from wood meal of different particle sizes, and D) shows time courses of TL, KL and ASL degradation in MW45. The reaction mixtures for both C) and D) contained 0.1% Tween 80. Values are presented as means ± standard deviation of triplicate samples. Values with the same letters were not statistically different ($P < 0.05$).

Fig. 2. Effects of multiple supplementations with co-oxidants on the MnP catalyzing delignification reaction. These reactions contained different combinations of the following co-oxidants: 0.1% Tween 80 (T80), 3 mM linoleic acid (LA), 3 mM reduced glutathione (GST) and 3 mM 1-hydroxybenzotriazole (HBT). A) indicates lignin degradation for each reaction. B) indicates lignin content in residual wood after a 24 h enzymatic reaction with MW150. Values are presented as means ± standard deviation of triplicate samples. Values with the same letters were not statistically different ($P < 0.05$).

Fig. 3. Lignin degradation in MnP/co-oxidants reaction under different conditions. Effect of (A) particle size of beech wood meal, (B) cellulase supplementation, (C) multi-step reaction and (D) wood species (D) are shown. Delignification reactions were performed against WM45 (B) and BMW (C and D) for 48 h. Values are presented as means ± standard deviation of triplicate samples. Values with the same letters were not statistically different ($P < 0.05$).

Fig. 4. Lignin degradation (A) and glucose yields (B) from beech MW45 following enzymatic delignification and saccharification. The reactions contained 20 and 60 FPU cellulase and either of two co-oxidants: 0.1% Tween 80 (T80) or a mix of 4 types of co-oxidants (4med). The composition of the control was same as for the 4med reaction but excluding MnP. Values are presented as means ± standard deviation of triplicate samples. Values with the same letters were not statistically different ($P < 0.05$), and the asterisk indicates a significant difference between the two samples.

Table 1: Monomeric product yields from DFRC and ANBO analysis of raw and enzymatic delignified wood

	raw wood	1 cycle	3 cycle
lignin degradation (%)	-	10.7±1.2	13.6±0.9
DFRC			
S/G ratio	1.53±0.12	0.81±0.05*	0.80±0.04*
total monomer recovery (%)[a]	100±7.4	58.2±0.9*	49.9±1.6*†
Salc recovery (%)[a]	100±6.5	49.8±1.0*	36.7±1.7*†
Calc recovery (%)[a]	100±10.4	83.5±5.9*	70.0±2.5*†
NB oxidation			
S/V ratio	2.56±0.03	2.79±0.27	2.72±0.13
total monomer recovery (%)[a]	100±3.0	82.7±3.3*	80.3±2.8*
S-type monomers recovery (%)[a]	100±3.3	84.5±5.0*	81.6±2.8*
V-type monomers recovery (%)[a]	100±2.3	78.0±3.9*	76.8±1.2*

[a] recovery was calculated based on yield from raw wood.

* indicates significantly difference from raw wood ($P < 0.05$)

† indicates significantly difference from 1 cycle reaction ($P < 0.05$)

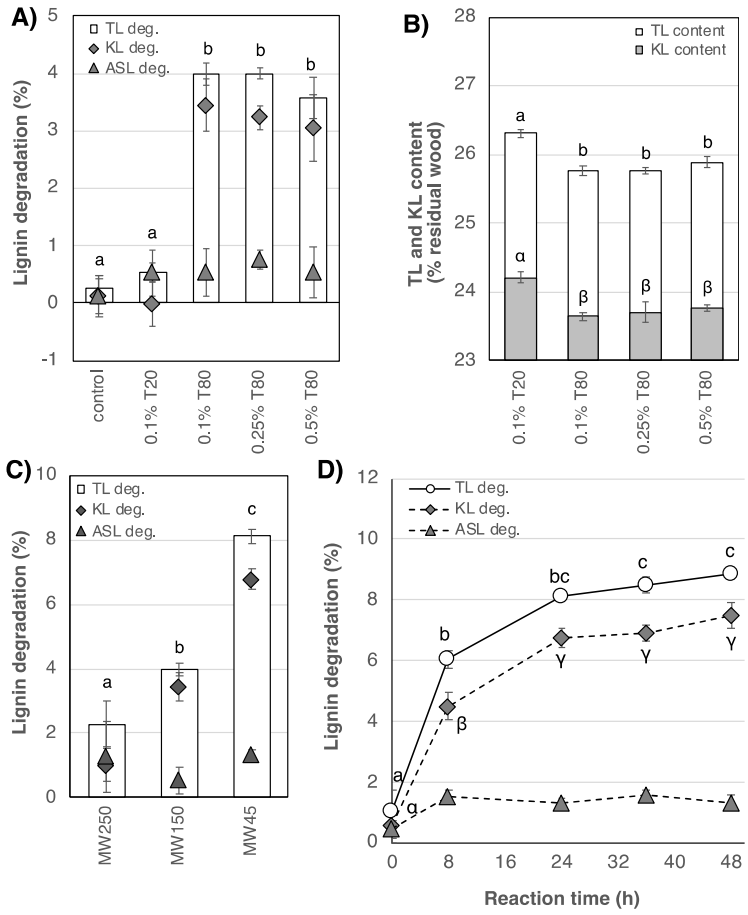

A)
TL deg.
KL deg.
ASL deg.
Lignin degradation (%)
control
0.1% T20
0.1% T80
0.25% T80
0.5% T80
B)
TL content
KL content
TL and KL content (% residual wood)
C)
MW250
MW150
MW45
D)
Reaction time (h)

Fig. 1

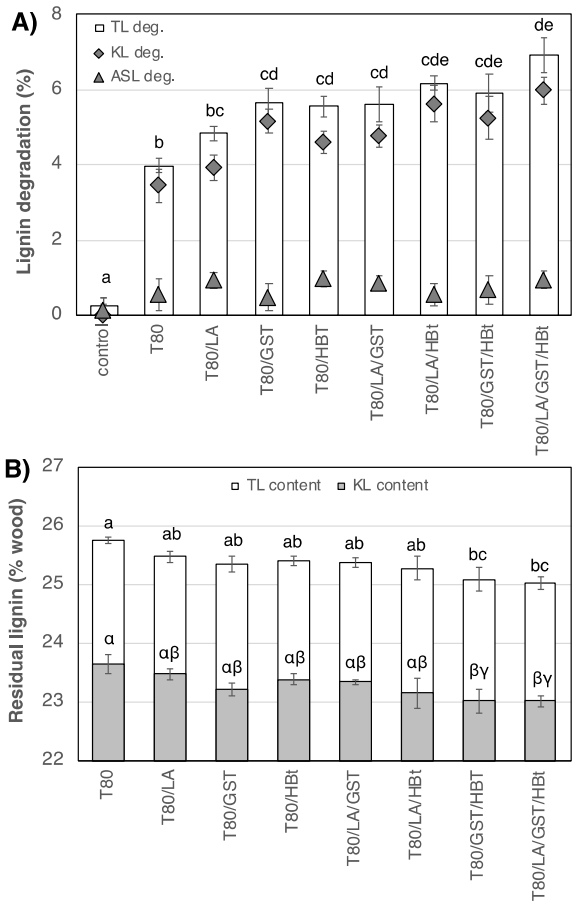
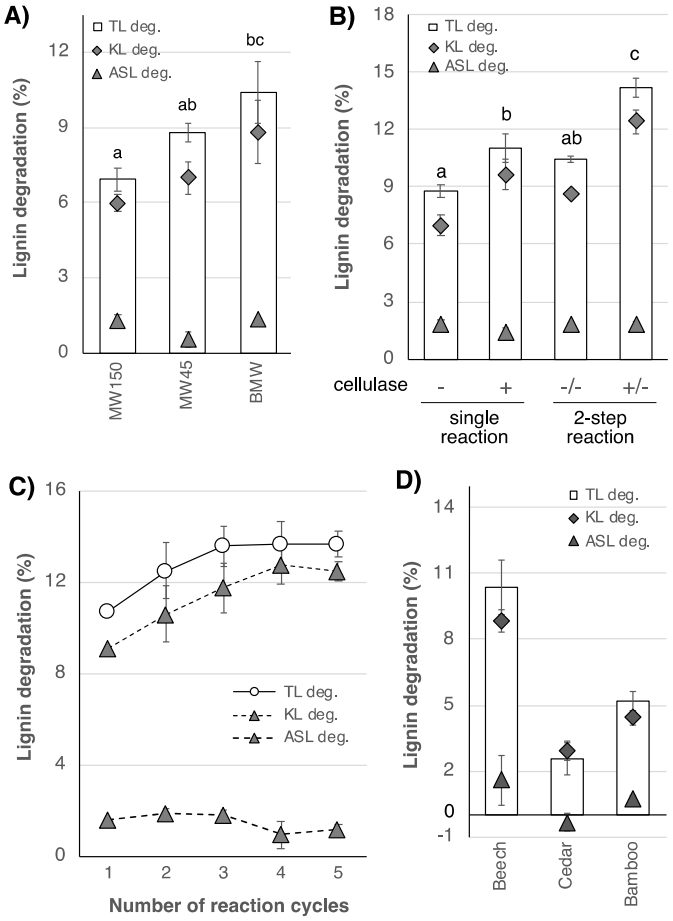

A)
TL deg.
KL deg.
ASL deg.
Lignin degradation (%)
0
2
4
6
8
a
b
bc
cd
cd
cd
cde
cde
de
control
T80
T80/LA
T80/GST
T80/HBT
T80/LA/GST
T80/LA/HBt
T80/GST/HBt
T80/LA/GST/HBt
B)
TL content
KL content
Residual lignin (% wood)
22
23
24
25
26
27
a
ab
ab
ab
ab
ab
bc
bc
α
αβ
αβ
αβ
αβ
αβ
βγ
βγ
T80
T80/LA
T80/GST
T80/HBt
T80/LA/GST
T80/LA/HBt
T80/GST/HBT
T80/LA/GST/HBt

Fig. 2



373

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377 Fig. 3

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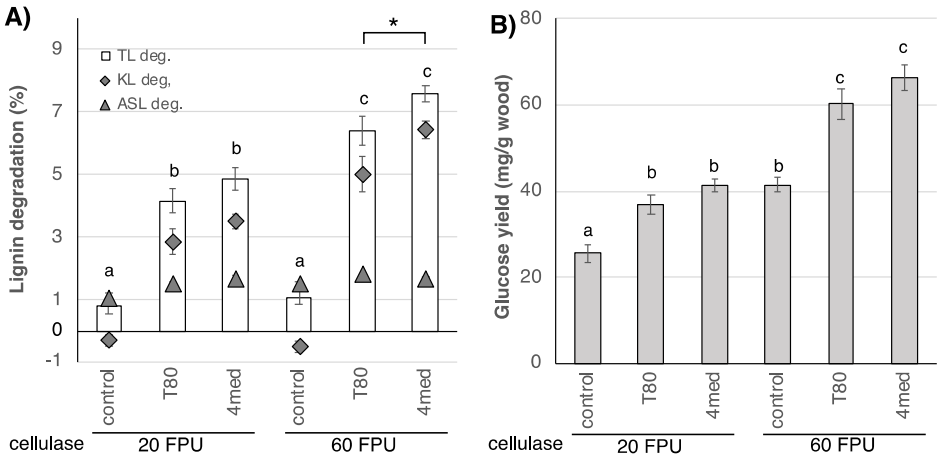
A)
Lignin degradation (%)
TL deg.
KL deg,
ASL deg.
control
T80
4med
cellulase
20 FPU
60 FPU
B)
Glucose yield (mg/g wood)

Fig. 4