Synergetic Depolymerization of Aspen CEL by Pyranose 2-Oxidase and Lignin-degrading Peroxidases

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Three enzymatic treatments were compared for the depolymerization of cellulolytic enzyme lignin (CEL) from aspen; these systems used pyranose 2-oxidase and lignin-degrading peroxidases. The "P" system was mainly composed of pyranose 2-oxidase, lignin peroxidase (LiP), and manganese peroxidase (MnP). Catalase and vitamin C were added to the P system to decompose H_2O_2 to control the H_2O_2 concentration. The system to which catalase was added was called the "C" system. The system to which catalase and vitamin C were added together was called the "V" system. Ultraviolet-visible (UV-Vis) spectra of supernatants after aspen CEL treatment by the P, C, or V system was used to monitor the amount of water-soluble lignin fragments that were generated, which increased with system treatment time. A gel permeation chromatography (GPC) analysis showed that after 12 h of system treatment, the molecular weight (M_w) of CEL was efficiently lowered; the maximum M_w reduction of aspen CEL was 20% when compared to the blank and control runs. The residual enzymatic activity of the supernatant after the CEL treatment by the P, C, or V system indicated that MnP and LiP activity for lignin degradation was dependent upon the H_2O_2 concentration. Therefore, it is advised that MnP and LiP be applied separately for effective lignin degradation.

Keywords: Synergetic degradation; Lignin; Pyranose 2-oxidase; Lignin peroxidase; Manganese peroxidase

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INTRODUCTION

Lignin is a heterogeneous biopolymer that is the most abundant aromatic material on Earth. It accounts for nearly 30% of the total organic carbon found in nature. Lignin is one of the few renewable feedstocks for the production of aromatic chemicals (Rahimi *et al.* 2014). However, the highly complex structure of lignin, in addition to its highly recalcitrant constitution that physically protects cellulose and hemicelluloses from hydrolysis degradation in the plant cell wall, has so far hampered many efforts to increase its application value (Bruijnincx and Weckhuysen 2014). Lignin depolymerization is an important process to efficiently utilize lignin for high-value products; such processes could provide a source of low-molecular-mass aromatic feedstocks that are suitable for downstream processing into bio-based materials in pharmaceutical and chemical industry (Ragauskas *et al.* 2014; Rahimi *et al.* 2014). Lignin bio-depolymerization can provide a green, mild, and energy-saving route for the production of low-molecular-mass aromatic feedstocks.

Lignin is degraded by a narrower range of microbes when compared to other major biopolymers found in nature. White rot fungi degrade lignin more rapidly and extensively

than other microbial groups (Camarero *et al.* 2014; Rouches *et al.* 2016). Meanwhile, the structural features of lignin require the biosystems responsible for initial depolymerization to be extracellular, nonspecific, and non-hydrolytic (Kirk and Farrell 1987). It is yet to be discovered the extracellular enzymes that are related to lignin biodegradation that are secreted by the white rot fungi, and contain lignin peroxidase (LiP), manganese peroxidase (MnP), laccase, H_2O_2-generating enzyme, and lignin fragment radicals reductase. In addition, some small molecular mass mediators, *e.g*., veratryl alcohol, oxalate, Mn^{3+}-Mn^{2+} and/or Fe^{3+}-Fe^{2+}, are also involved in the lignin degradation because the size of the secreted enzymes are too large to diffuse into the fiber walls (Janusz *et al.* 2017). In addition to lignin degradation, the lignin extracellular degrading enzyme discovered also can degrade many persistent organic pollutants to remove the environmental pollution caused by them.

Lignin peroxidase is able to depolymerize lignin when H_2O_2 is present (Odier *et al.* 1988). When the way of H_2O_2 supply changed from the interval supply to the continuous supply *via* a peristaltic pump or glucose oxidase, lignin degradation was observed (Hammel and Moen 1991; Wariishi *et al.* 1991; Hammel *et al.* 1993). The actions of glucose oxidase and pyranose 2-oxidase for reducing quinoid intermediates improved the depolymerization of kraft (sulfate) lignins by laccase (Ai *et al.* 2014). Thus, *in vitro*, the extracellular bio-depolymerization of lignin is an oxidative process controlled by the reductive reaction and/or the supply of H_2O_2.

Pyranose 2-oxidase can also generate H_2O_2 while reducing quinoid groups. In this research there was an attempt to achieve synergistic effects in the degradation of cellulolytic enzyme lignin (CEL), which was obtained from aspen, by pyranose 2-oxidase, lignin peroxidase (LiP), and manganese peroxidase (MnP). To better understand the factors affecting decomposition, three systems were compared. The first system was mainly composed of pyranose 2-oxidase, lignin peroxidase (LiP), and manganese peroxidase (MnP). Catalase and vitamin C were added to the first system to control the H_2O_2 concentration. The system that catalase was added into the first system formed the second system. The system that catalase and vitamin C were all added into the first system was the third system.

EXPERIMENTAL

Materials

All solvents used in this study were analytical reagent grade and used as purchased without further purification unless otherwise stated. The tetrahydrofuran (THF) used was chromatographic grade. The 1,4-dioxane, THF, pyridine, and acetic anhydrate were distilled prior to being used. Catalase, veratryl alcohol, and 2,6-dimethoxyphenol (2,6-DMP) were purchased from Sigma (Sigma-Aldrich, Shanghai, China). Other chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Cellulase was provided by Novozymes (Beijing, China); it contained 202 IU/g xylanase, 308 IU/g *β*-D-glucosidase, 0.407 IU/g cellobiohydrolase, 99 IU/g endoglucosidase, and 608 IU/g filter paper unit (FPU) activity.

Lignin

Cellulolytic enzyme lignin (CEL) was isolated from aspen wood according to the method of Zhang *et al.* (2010). The weighted-average molecular weight (*Mw*) of the CEL obtained was approximately 16,000 g/mol.

Enzymes

Pyranose oxidase was obtained from *Irpex lacteus* dft-1. Its purification was completed in accordance to the method of Ai *et al*. (2014). The LiP and MnP were obtained from *Phanerochaete chrysosporium* ME446. The cultivation and purification of these enzymes was performed in accordance to the method reported by Tien and Kirk (1988), with the exception that 3 mM veratryl alcohol was used instead of 40 mM. Nine proteins detected from the Mono-Q column had absorbance at 409 nm. According to the elution sequence and Kirk's naming, there was no fifth protein (H5), and there were H1, H2, H3, H4, H6, H7, H8, H9, and H10. The protein designated H1, H2, H6, H7, H8, H9, and H10 had LiP activity. H3 and H4 had MnP activity. The mixed MnP (H3 and H4) and the mixed LiP (H1, H2, H6, H7, H8, and H10) were used in the experiments of this study.

Treatment of CEL by pyranose 2-oxidase and lignin degrading peroxidases

The CEL (10 mg) was added to a 50-mL sterilized conical centrifuge tube. The system designated as the P system contained the following items added to 10 mL of a 4.5 pH buffered solution (20 mM sodium succinate): 3 IU LiP, 4 IU MnP, 0.3 IU pyranose 2-oxidase, 0.15 IU inactivated catalase, 4 μmol veratryl alcohol, 5 μmol Mn^{2+}, 500 μmol lactate acid, and 500 μmol glucose. The system designated as the C system had the same components of the P system except that activated catalase was used in place of the inactivated catalase. The system designated as the V system contained the same components as the C system except that the V system contained 2 μmol of vitamin C. Pyranose 2-oxidase was inactivated in the control experiments, which had the same components as the P system. All of the enzymes were inactivated in the blank experiments, which had the same components as the P system. The veratryl alcohol, Mn^{2+}, lactate acid, and glucose were filtered and sterilized. The 4.5 pH buffer of sodium succinate was sterilized in an autoclave. There were three replicates for every experiment.

A 50-mL conical centrifuge tube was sealed with a silicone plug into which a 6-mm diameter syringe needle was inserted as the oxygen outlet, and a 3.2-mm male luer straight joint was inserted as the oxygen inlet (Fig. 1). A syringe needle needle-head and the Pagoda interface of the inserted male luer straight joint were facing towards the inside of the centrifuge tube. The Pagoda interface of the male luer straight joint was connected with a 9-cm-long and 3-mm inner diameter silicone tube. The free end of the silicone tube was connected to a 10-μL suction head whose cusp was submerged 1.8 cm into the liquid of the conical centrifuge tube. The other end of the male luer straight joint was connected a 20-cm-long and 3-mm inner diameter silicone tube. The silicone tube was connected to the 20-outlet gas discharge whose main inlet was connected to the oxygen cylinder with an 8-mm inner diameter silicon tube. All of the silicon tubes and the gas discharge were sterilized in an autoclave.

These 50-mL conical centrifuge tubes with the silicone plug and connected tubes were placed in a shaking water bath at 40 °C at an oscillation rate of 120 rpm. The rate of oxygen introduced into the tube was adjusted to one bubble per second. One tube of the blank, control, P system, C system, and V system were removed from the shaking bath at 3, 6, 12, and 24 h intervals. The remained outlets of the gas discharge were sealed after the samples were removed.

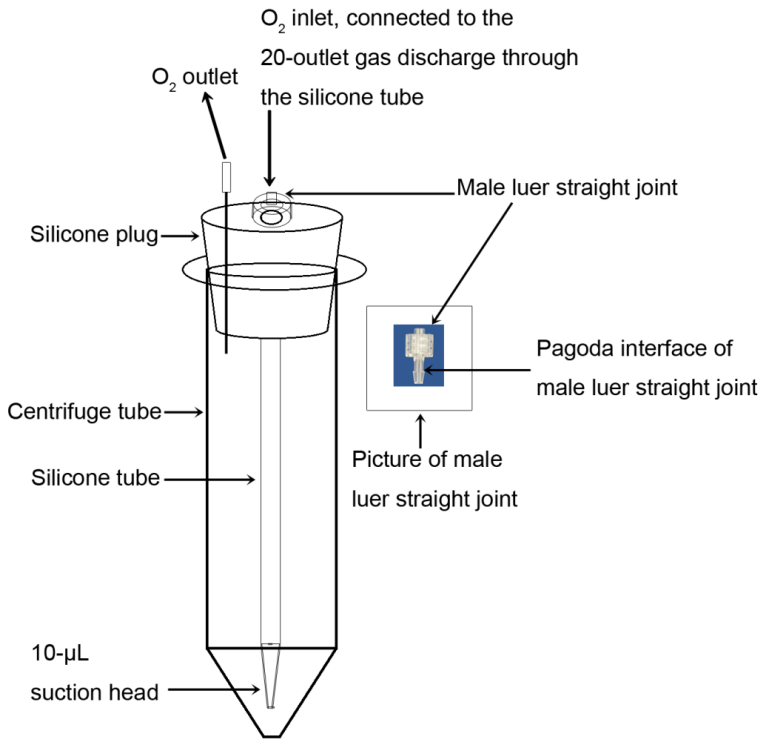

Fig. 1. Schematic of the CEL degradation device used in this study

The sample that was removed from the shaking water bath was centrifuged at 10,000 rpm at 4 °C. The resulting supernatant was transferred using a pipette into a 15-mL centrifuge tube. Then, the supernatant was kept at 4 °C for residual enzyme activity determinations and ultraviolet-visible (UV-Vis) scanning. The solid lignin was washed three times with double distilled water; afterwards, the washed solids were dried under vacuum.

Methods

Determination of residual enzyme activity

The LiP activity was measured spectrophotometrically during the oxidation of veratryl alcohol to veratraldehyde in the presence of H_2O_2 in a 2.5 pH sodium tartrate buffer (250 mM) at 310 nm wavelength ($\varepsilon_{310\ nm}$ = 9,300 $M^{-1}cm^{-1}$) (Haq *et al.* 2016). The MnP assay was based on the oxidation of 2,6-dimethoxyphenol to 3,3',5,5'-tetramethoxy-diphenoquinone in the presence Mn^{2+} and H_2O_2 in a 4.5 pH sodium lactate buffer (100 mM) at 469 nm wavelength ($\varepsilon_{469\ nm}$ = 49,600 $M^{-1}cm^{-1}$) (Ng *et al.* 2015). Pyranose 2-oxidase activity was determined by monitoring the formation of H_2O_2 with a peroxidase-catalyzed chromogenic reaction with 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) at 420 nm wavelength ($\varepsilon_{420\ nm}$ = 43,200 $M^{-1}cm^{-1}$) (Ai *et al.* 2014). Catalase activity was measured spectrophotometrically by monitoring the H_2O_2 breakdown at 240 nm wavelength ($\varepsilon_{420\ nm}$ = 43.6 $M^{-1}cm^{-1}$) (Ogunlana *et al.* 2012).

UV-Vis spectroscopy

The UV-Vis absorption spectra of the centrifuged supernatants were recorded over the range of 190 to 700 nm (at a 1 nm resolution) using a spectrophotometer (Agilent 8453;

Agilent Technologies, Santa Clara, CA, USA). The rectangular quartz cuvette used in the spectrophotometer had a path length of 1 cm.

Gel permeation chromatography (GPC)

The dried solid lignin was dissolved in 250 µL of distilled pyridine and 250 µL of distilled acetic anhydride for 72 h under darkness. The lignin acetylation was performed with slight shaking at room temperature. The acetylated lignin was precipitated in 7 mL diethyl ether and washed three times with diethyl ether. The acetylated lignin was finally dried and kept under vacuum at 30 °C.

The molecular weight distributions of the treated CELs were measured using a Waters 1515-2414 gel permeation chromatograph (Waters, Milford, MA, USA) with a Styragel® HT THF column. The dried and acetylated lignin was dissolved in tetrahydrofuran (4 mg/mL) overnight. The solution was filtered through a 0.22-µm polytetrafluoroethylene(PTFE) membrane to avoid plugging the GPC column. A volume of 50 µL of the filtrate was injected. The temperature of the column was 39 °C. Tetrahydrofuran was used as the mobile phase with a flow rate of 1 mL/min. A differential refractometer (RI; Waters, Milford, MA, USA) was used as the detector at the end of the column. Polystyrene standards were used to generate a weighted-average molecular weight calibration curve.

RESULTS AND DISCUSSION

UV-Vis of Supernatant Generated from the CEL Treated by Pyranose 2-Oxidase and Lignin Degrading Peroxidases

The resolutions obtained from the recorded UV spectra (190 nm to 240 nm) of the supernatants were too low to obtain discernible differences among the enzymatic degradation treatments. The absorbance level obtained from the recorded visible spectra (400 nm to 700 nm) of the supernatants were too similar to obtain discernible differences among the enzymatic degradation treatments. The near ultraviolet spectra regions (240 nm to 400 nm) for the various degradation treatments are shown in Fig. 2. All spectra had a strong absorbance band centered at 280 nm. The absorbance at 280 nm of the supernatant from the control in which lignin was only treated by peroxidases; these spectra peaks were higher than those of the blank (no active enzymes present). The absorbance at 280 nm of the supernatants obtained after lignin treatment by the P, C, and V enzymatic systems were all higher than the supernatant of the control. The strong absorbance centered at 280 nm is assigned to the non-conjugated phenolic structures of lignin (Xu *et al.* 2006; Bahl *et al.* 2014). The amount of protein added in all systems was the same. Thus, the differences observed in the absorbance around 280 nm were attributed to new phenolic structures being generated during the enzymatic depolymerization of the CEL by the various systems. This indicated that pyranose 2-oxidase and peroxidases can synergistic-ally cleave the β-O-4 linkages in lignin to create more unconjugated phenolics in the solubilized lignin. All supernatants from the three enzymatic systems, except for the C system at 3 h treatment, had a shoulder peak around 310 nm (Figs. 2A through 2D). This shoulder peak may associate with veratraldehyde, which evolves from the oxidation of veratryl alcohol by LiP in the lignin treatment system. Likewise, this peak could be attributed to some lignin degradation products, such as *p*-coumaric, vanillic, ferulic, and sinapinic acid (Herrera and Luque de Castro 2005; Waliszewski *et al.* 2007; Lu *et al.* 2017; Soltani *et al.* 2018).

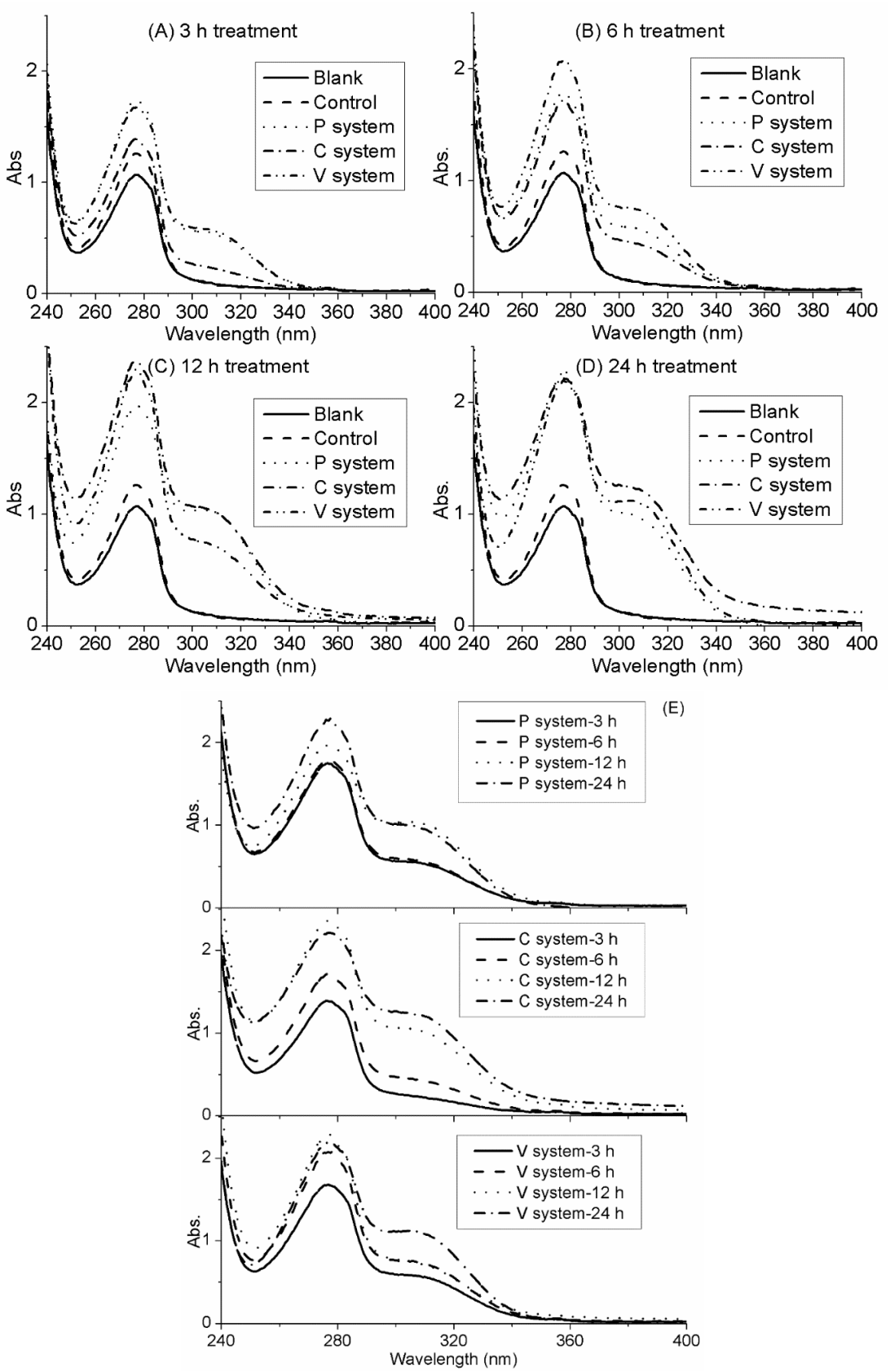

Fig. 2. Near ultraviolet spectra of CEL supernatants after treatment by pyranose 2-oxidase-lignin degrading peroxidases systems

After 3 and 6 h of enzymatic degradation treatment, the absorbance at 280 and 310 nm of the supernatants from the P and V system were higher than the C system (

FigA and 2B). After 12 and 24 h of enzymatic degradation treatment, the absorbance at 280 and 310 nm of the supernatants from the P and V system was lower than the C system (

FigC and 2D). The absorbance at 280 nm of the supernatant from the P system increased with increasing treatment time, as well as the absorbance at 310 nm until 12 h (Fig. 2E). For the C and V systems, the absorbance at 280 nm of the supernatants increased over the 12 h of treatment, whereas the absorbance at 310 nm continued to increase during the 24 h of treatment (Fig. 2E). Hence, with increased enzymatic treatment time, the amount of the water-soluble lignin fragments produced from the depolymerization of CEL by the P, C, or V system also increased.

Molecular Weight of CEL After Pyranose Oxidase-lignin Degrading Peroxidases Treatments

Figures 3 and 4 indicate that the M_n and M_w of the CEL in the blank and control treatments were unchanged as the treatment time increased. The M_n and M_w of the CEL from the control were lower than the blank. This observation suggested that the control treatment depolymerized CEL. A previous investigation reported that lignin peroxidase can oxidize Mn^{2+} to Mn^{3+} in the presence of veratryl alcohol, oxygen, and lactic acid (Popp *et al.* 1990). The generating Mn^{3+} could oxidize phenolic lignin structures, which degraded the CEL. This oxidative degradation reaction could explain the decrease of the M_n and M_w values of the CEL in the control *versus* the blank treatment.

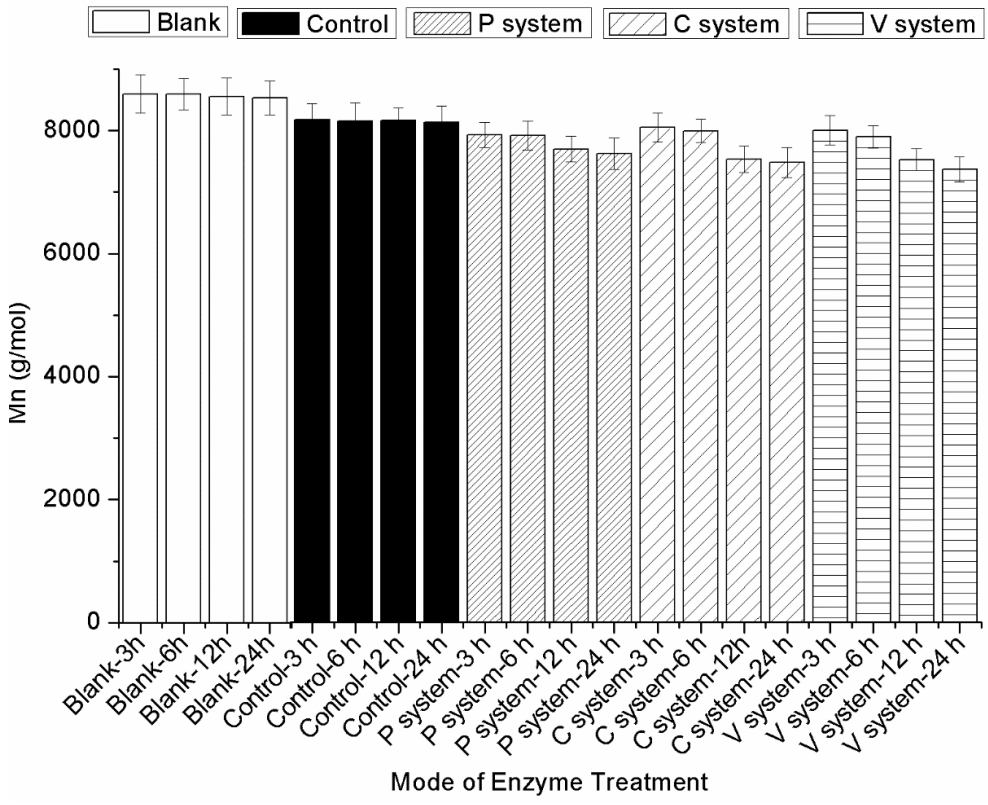

Fig. 3. Number-average molecular weight (M_n) of the CEL after various pyranose 2-oxidase-lignin degrading peroxidases treatments

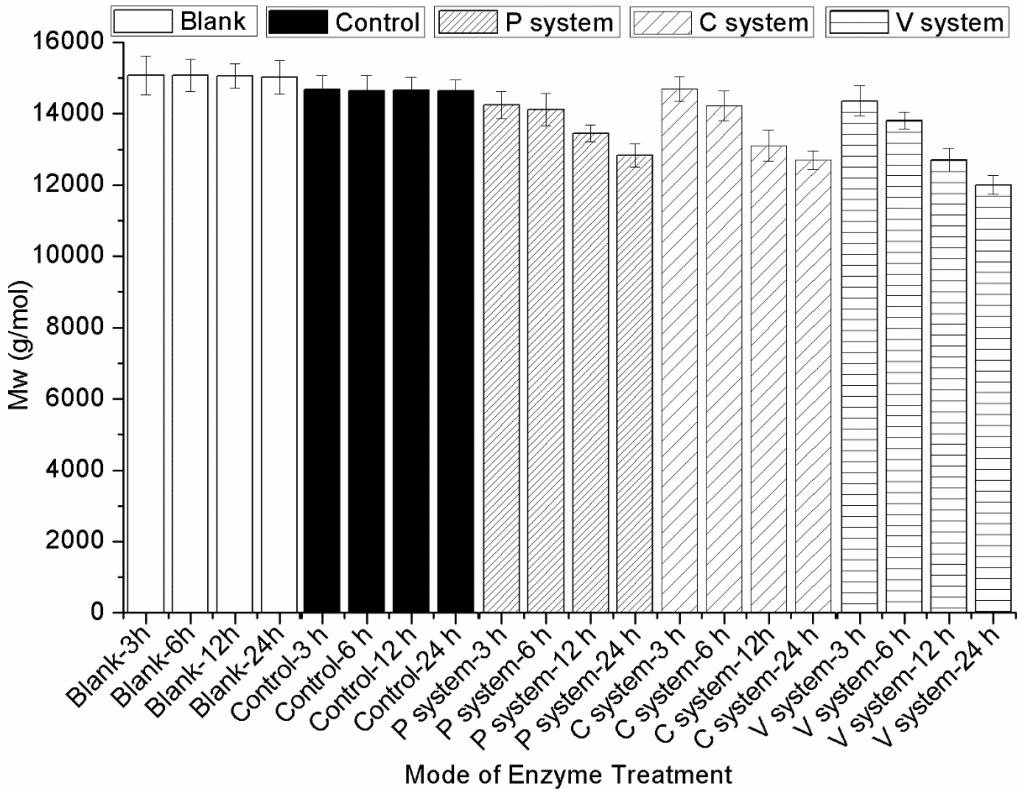

Fig. 4. Weighted-average molecular weight (M_w) of the CEL after various pyranose 2-oxidase-lignin degrading peroxidases treatments

In Figs. 3 and 4 the histograms became shorter within P, C, and V groups, which indicates an enzymatic treatment effect increasing with increasing lengths of time. When compared to the corresponding control, the P, C, or V system caused the M_n and M_w values to decrease with increasing treatment time. The reduction rate of the M_n and M_w values was fastest after 12 h (Figs. 3 and 4). After 3 and 6 h of enzymatic treatment, the M_w and M_n values of the CEL treated by the C system were slightly higher than the CEL treated by the P or V systems (Figs. 3 and 4). Consequently, the amount of the water-soluble lignin fragments produced from the C system was lower than those from the P or V systems; this observation was consistent with the observed UV-Vis absorption of the supernatants after P, C, or V treatments (Fig. 2). The M_w and M_n of the CEL after the V system for 24 h decreased 20% and 14%, respectively, when compared to the blank. After 12 and 24 h of treatment, the M_w and M_n of the CEL by the V system were lower than that of the C system, and the M_w and M_n of the CEL obtained from the C system was lower than the P system (Figs. 3 and 4). This observation indicated that after 12 h of enzymatic degradation treatment, the addition of catalase to the C system enhanced the synergetic depolymerization of CEL by lignin peroxidases and pyranose 2-oxidase. Vitamin C addition to the V system further enhanced this effect. It has been previously reported that vitamin C can contribute to the degradation of a synthetic lignin polymer by *P. chrysosporium* (Hong *et al.* 2017).

Residual Enzyme Activities After Pyranose 2-Oxidase Lignin-degrading Peroxidases Treatments

The residual enzyme activities in the supernatants after the different pyranose 2-oxidase-lignin peroxidases system treatments are shown in Fig. 5. The blank treatment exhibited no residual enzymatic activities (*i.e.*, LiP, MnP, pyranose 2-oxidase, and catalase) (Fig. 5). This observation was consistent with the fact that all of the enzymes used in the blank treatment were deactivated. In comparison to the initial enzyme activity added to the control (*i.e.*, 0.3 IU/mL LiP and 0.4 IU/mL MnP), there was lower residual LiP activity and higher residual MnP activity after the treatment. During the course of the control treatment, the residual LiP activity remained almost unchanged, whereas the residual MnP activity gradually decreased (Figs. 5A and 5B).

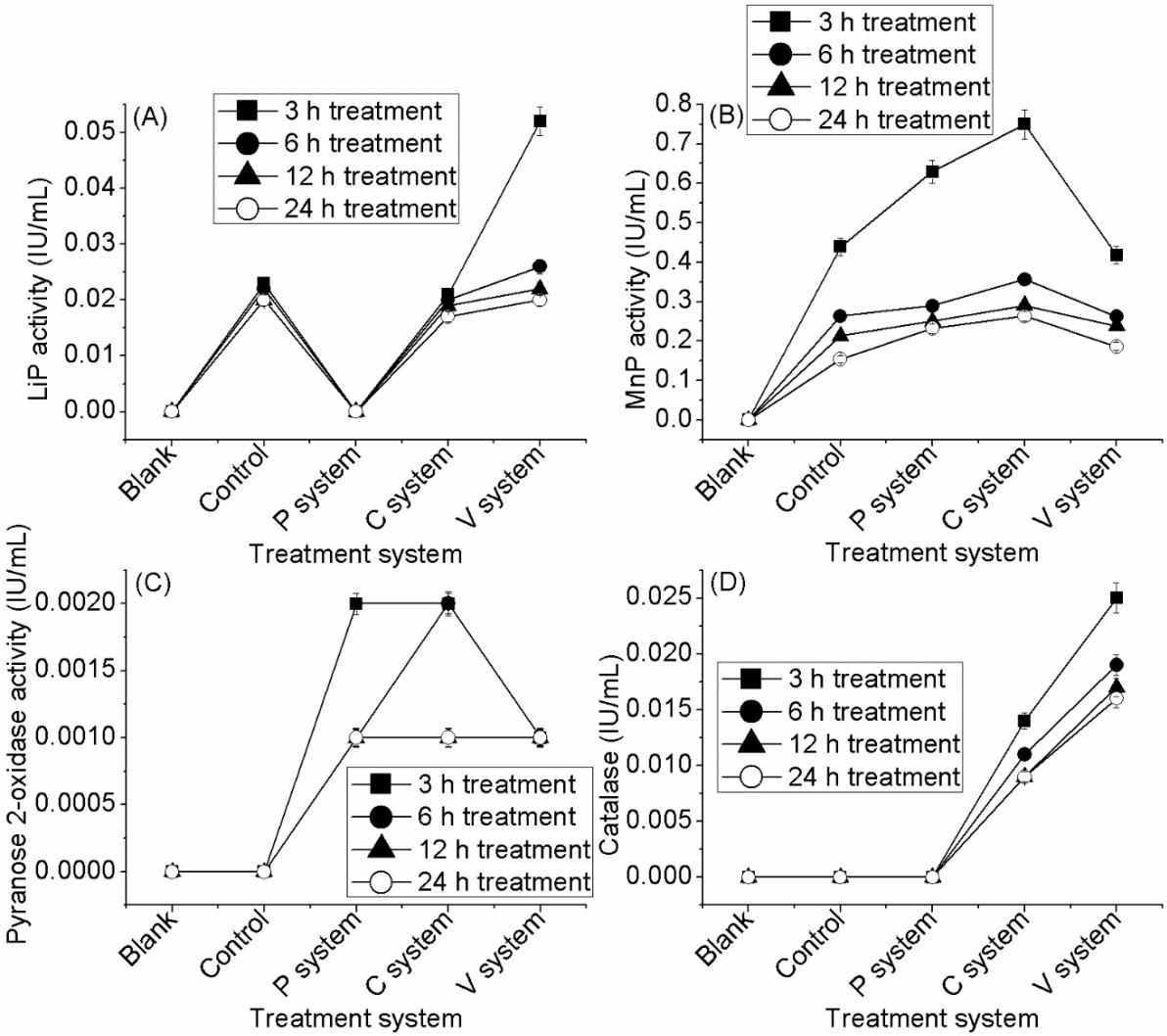

Fig. 5. Residual enzyme activities of pyranose 2-oxidase lignin-degrading peroxidases, and catalase for P, C, and V systems *versus* blank and control treatments

For the P system, the residual LiP activity was undetected during the entire treatment time (Fig. 5A). However, there was higher residual MnP activity and lower residual pyranose 2-oxidase activity when compared to the initial enzymatic conditions of the P system (Figs. 5B and 5C). The residual MnP activity in the P system was higher than that of the control at the same treatment time (Fig. 5B). In the C system, the residual LiP activity was lower, the residual MnP was higher, the residual pyranose 2-oxidase was lower, and the residual catalase was higher than the initial conditions at 3 h, 6 h, 12 h, and 24 h (Figs. 5A to 5D). Similarly, the residual MnP and catalase activities in the C system decreased as the treatment time increased. There was higher residual LiP activity in the V system *versus* the control, the P system, and the C system. In addition, the V system had higher MnP, lower pyranose 2-oxidase, and higher catalase activities after 3 h, 6 h, 12 h, and 24 treatment times *versus* the initial conditions. The residual MnP and catalase activities gradually decreased with the V system as the treatment time increased.

Pyranose 2-oxidase produced excess hydrogen peroxide, which inactivated the LiP (Wong 2009). Hence, there was no LiP activity in the P system (Fig. 5A). However, the M_w value of the CEL decreased with the P system after 24 h of treatment. This observation indicated that the CEL depolymerization was not caused by the action of LiP. In this case, CEL depolymerization was mainly attributed to the effect of MnP. The dose of pyranose 2-oxidase added to the P system was appropriate for MnP (Fig. 5B) because the activity of MnP in the P system after 3 h treatment was higher than the initial amount. The effect of active pyranose 2-oxidase on MnP is same as MnP with glucose oxidase (Ansari *et al*. 2016, 2017). In the C system, LiP and MnP of 3 h treatment was 0.021 IU/mL and 0.75 IU/mL. When compared to the initial added LiP and MnP activity, catalase restored LiP activity to 7% after 3 h and made the MnP activity to increase 1.87 times. It indicated that the inactivation of LiP was partially mitigated by the catalytic decomposition of H_2O_2 by added catalase and the activity of MnP in the C system was further enhanced (Fig. 5B). Thus, the M_w of the CEL after the C system treatment for 12 and 24 h was lower than that of the P system treatment. In the V system, LiP activity increased 17% after 3h treatment. Catalase and vitamin C allowed more of the LiP to remain in its active form, although the activity of MnP after 3 h treatment reverted to the same level as the control. The M_w of the CEL after the V system treatment for 12 and 24 h decreased even further.

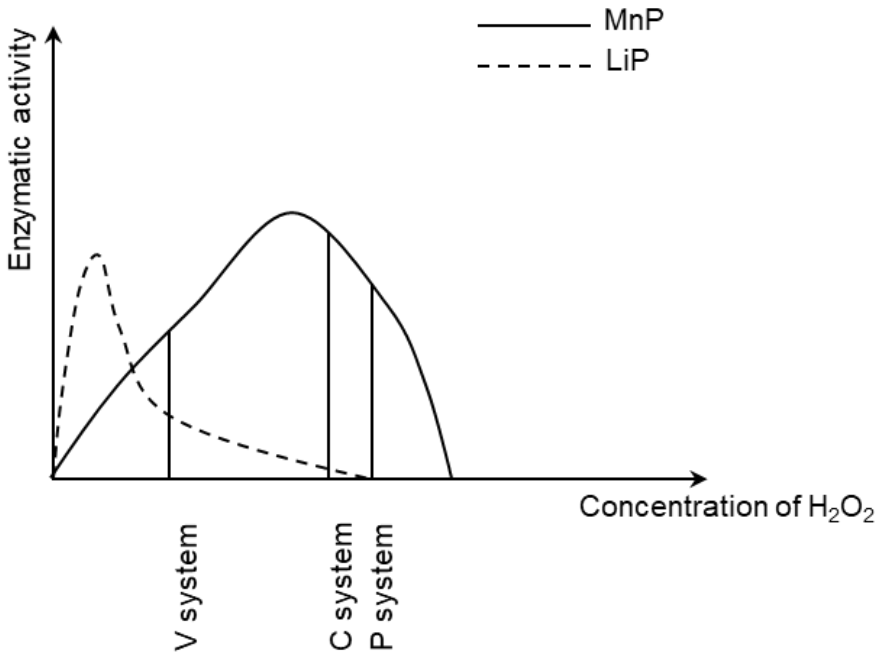

Fig. 6. Schematic diagram of presumed activity changes to LiP and MnP with the concentration of H_2O_2

Catalase can catalyze H_2O_2 decomposition and decrease its concentration in the system as it is generated by pyranose 2-oxidase. In this experiment the lower H_2O_2 concentration in the C system *versus* the P system enhanced the activities of LiP and MnP for the C system (Fig. 5). Vitamin C converted H_2O_2 to water and oxygen, which reduced the concentration of H_2O_2 in the V system. Hence, high LiP activity was more efficiently retained, but the activity of MnP was reduced (Fig. 5). This may have been attributable to the fact that the optimum amount of H_2O_2 produced from pyranose 2-oxidase that is needed for LiP and MnP to depolymerize lignin and for the peroxidases to remain active may have been appreciably different from one another (Fig. 6). Consequently, to construct an efficient system of lignin depolymerization, when pyranose 2-oxidase is used as the generator of H_2O_2, it is advised that LiP and MnP should be separately applied to lignin depolymerization. The ratio of MnP or LiP to pyranose 2-oxidase for lignin depolymerization needs to be further studied to determine the optimum value. In addition, the activities of MnP and LiP in relation to the concentration of H_2O_2 need to be investigated to determine the most favorable conditions.

CONCLUSIONS

1. Pyranose 2-oxidase and lignin-degrading peroxidases efficiently depolymerized aspen CEL during 24 h of treatment.
2. Compared to the initial added enzymatic activity, catalase restored LiP activity to 7% and MnP activity increased by 1.87 times after 3 h treatment in the C system.

3. Compared to the initial added enzymatic activity, catalase and vitamin C restored LiP activity to 17% and had no capacity to activate MnP after 3 h treatment in the V system.
4. The optimum amount of H_2O_2 produced from pyranose 2-oxidase that is needed for LiP and MnP to depolymerize lignin was appreciably different from one another. To construct an efficient system of lignin depolymerization, when pyranose 2-oxidase is used, it is advised that LiP and MnP should be separately applied to lignin depolymerization.

ACKNOWLEDGEMENTS

This work was supported by the Director's Fund of the State Key Laboratory of Bio-Based Materials and Green Papermaking at Qilu University of Technology (Grant No. 030803138612) and was helped with providing the *Irpex lacteus* dft-1 and *Phanerochaete chrysosporium* ME446 by the Microbial Technology Institute of Shandong University.

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Article submitted: November 21, 2018; Peer review completed: February 4, 2019;
Revised version received and accepted: February 22, 2109; Published: March 8, 2019.
DOI: 10.15376/biores.14.2.3481-3494