H_2O_2 can Increase Lignin Disintegration and Decrease Cellulose Decomposition in the Process of Solid-State Fermentation (SSF) by *Aspergillus oryzae* Using Corn Stalk as Raw Materials

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H_2O_2 is both bactericidal and the main oxidant responsible for lignin degradation reaction catalyzed by manganese peroxidase (MnP) and lignin peroxidase (LiP). Thus, H_2O_2 treatment of corn stalk and the implementation of solid-substrate fermentation (SSF) is possible to increase the removal rate of lignin from stalk in the process of SSF and after SSF, while avoiding the need to sterilize the raw materials. To demonstrate this approach, SSF was initially carried out using corn stalk pretreated with different concentrations of H_2O_2 as a substrate. *A. oryzae* was found to grow well in the 3% H_2O_2-pretreated corn stalk. H_2O_2-pretreated corn stalk showed increased MnP and LiP synthesis and disintegration of lignin, but inhibited cellulase synthesis and cellulose degradation. Production of the SSF (200 g) on the 10th day was hydrolyzed in the presence of additional 600 mL different concentration of H_2O_2 aqueous solution. The total removal of lignin (73.15%) of hydrolysis for 10 h at 3% H_2O_2 solution was highest and far higher than that at the 12th day, as achieved by conventional SSF. Applying this strategy in practice may shorten the time of lignin degradation, increase the removal of lignin, and decrease the loss of cellulose. Thus, this study has provided a foundation for further study saccharification of corn stalk.

Keywords: Aspergillus oryzae; Solid-substrate fermentation; Hydrogen peroxide; Biofuel; Hydrolysis

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INTRODUCTION

World production of corn, one of the major cereal crops, exceeds 840 million tons per annum, which yields about 3.3 billion tons of stalks (Kopania *et al*. 2012). These and other agricultural residues exiting as a waste stream from commercial crop processing plants have little inherent value and have traditionally constituted a disposal problem. These materials, as other kinds of straws, represent an abundant, inexpensive, and readily available source of renewable lignocellulosic biomass. However, their utilization as a carbohydrate source for glucose and ethanol production has been severely hampered by the low efficiency of converting the polysaccharide portion of the residue into monomeric

sugars by organisms and enzymes (Gould 1989). The lignin is one of the main factors contributing to the low efficiency of conversion.

Lignin is a highly irregular and insoluble polymer that is chemically bound to hemicellulose by covalent bonds, forming lignin-carbohydrate complexes enwrapping cellulose in the plant cell wall (Péňrez *et al.* 2002). Lignin is not only difficult to biodegrade, but also acts as a physical barrier between cellulose and its degradation enzyme to hamper its degradation (Digabel and Avérous 2006; Huang *et al.* 2006; Lopez *et al.* 2006; Malherbe and Cloete 2002; Tuomela *et al.* 2000; Vikman *et al.* 2002).

Biological treatment, in which the lignin in the stalk is degraded and digested by microorganisms, has gained more attention and will undoubtedly be applied in lignin degradation. Solid-substrate fermentation (SSF) has become popular recently as a biological treatment. It is simply defined as a process whereby an insoluble substrate in composts, with sufficient moisture but absent or nearly absent of free water, can be degraded by different microorganisms (Mitchell and Lonsane 1993). SSF is characterized by its higher volumetric productivity, simplicity, and lower energy requirement; it easily meets the aeration requirements, resembles the natural habitat of some fungi and bacteria, and has easier downstream processing without producing wastewater. Therefore, SSF is appropriate for the biological pretreatment of stalk biomass. However, the following two impediments must be overcome before SSF is practical to use to pretreat stalk for ethanol production. First is how to sterilize the stalks. Because the character of stalk is large volume and small density, steam sterilization will significantly increase the cost of production to exceed the price of ethanol. Second is how to simultaneously reduce the degradation of cellulose but increase the disintegration of lignin in stalk to provide more sugar for the subsequent saccharification and fermentation. Although the lignin can also achieve this objective by the water-washing, the waste water resulted from the process water-washing contains high levels of organic materials, leading to environmental pollution by being direct discharged without being treated. In other words, such effluent must be treated before being discharged.

Hydrogen peroxide is bactericidal at high concentrations and has been used extensively in medicine and sanitation. It is also the simplest strong peroxider (a compound with an oxygen-oxygen single bond). The oxidizing capacity of H_2O_2 is so strong that it is considered a highly reactive oxygen species. In addition, H_2O_2 is easy to manipulate and apply, flexible to use as a reagent, convenient to transport and store, and has low volatility. It is well known that H_2O_2 is also a substrate of ligninolytic enzymes; therefore, by using H_2O_2 to pretreat the raw stalk materials it is possible to increase the removal rate of lignin in the process of SSF and benefit to subsequent saccharification and fermentation.

The white-rot fungus *Phanerochaete chrysosporium* is the most commonly used model organism in lignin biodegradation studies. This filamentous fungus possesses a unique ability to efficiently degrade lignin to CO_2 (Hofrichter 2002). However, the disadvantages of the strain are that it cannot grow in the presence of a high concentration of hydrogen peroxide (H_2O_2), which is one of the substances of the lignin degradation reaction catalyzed by ligninase. This fungal strain can secrete ligninase to degrade lignin in a carbon and nitrogen limiting medium, but meanwhile also produces cellulase to degrade cellulose, which is the substrate to generate ethanol.

Aspergillus oryzae, a deuteromycotina fungus that secretes many kinds of enzymes including protease, amylase, cellulase, and phytase (Sun *et al.* 2007), is generally recognized as safe by the Food and Drug Administration (FDA) and Association of American Feed Control Officials (AAFCO) (Marui *et al.* 2010; Matsumura *et al.* 2004). It is extensively used in wastewater disposal and bioremediation in the food, feed, kojic acid, and brewery fermentation industries (Kammoun *et al.* 2008). Tung *et al.* (2004) used *A. oryzae* to treat the wastewater from cassava starch processing (CSP), with 90% chemical oxygen demand (COD) of wastewater ultimately removed under an optimized treatment condition. Bhalerao and Puranik (2009) studied *A. oryze* ARIFCC1054 degradation of monocrotophos (MCP) and found that the strain possessed phosphatase activity and could be used to renovate MCP-contaminated soil and treat the aqueous wastes. Meng *et al.* (2010) isolated *A. oryzae* 112822 from tobacco leaves; such treatment degraded the available nicotine, and the authors were the first to elucidate a pathway to degrade nicotine in fungi. After treating straw in exploded stover with *A. oryzae*, the contents of cellulose and hemicellulose in the exploded and fermented corn stover (EFCS) decreased by 24.36% and 69.90%, respectively, compared to untreated stover and by 17.35% and 38.59%, respectively, compared to exploded stover (Chang *et al.* 2012).

In a previous study, we reported the isolation of the *A. oryzae* strain CGMCC5992 (GenBank accession number of its 28s rDNA sequence: KC291246-KC291247) that could degrade organic substances, including difficult-to-degrade chemicals, and reduce the COD of vinasse (Zhang *et al.* 2013). The present study introduces a new method to treat corn stalk with H_2O_2 and *A. oryzae*. The microorganisms in the stalk were killed, and the bacterial colloid was removed by the chemical action of H_2O_2. The lignin in the stalk was then degraded by catalysis of LiP from *A. oryzae* CGMCC5992.

EXPERIMENTAL

Materials

The corn stalk and corncob were purchased from a local farm. They were directly cut into pieces approximately 3 cm in length for further use without water-washing to avoid producing waste water, and then ground to pass through a 0.25-mm sieve, respectively. The composition (w/w) of corn stalk used was 35.9% cellulose, 34.9% hemicelluloses, 19.6% lignin, 3.6% protein, 3.5% ash, and 2.7% wax. All weights and calculations were performed on oven-dried (50 °C, 16 h) material. All chemicals used were of analytical or reagent grade.

Methods

Microorganism

A. oryzae CGMCC5992 isolated from the sludge of the Yudai River at Jiangsu University and stored in the China General Microbiological Culture Collection Center was used in this study. It was grown on the potato dextrose agar (PDA) slants at 28 °C for 4 days, then stored at 4 °C and passaged every 7 to 9 weeks.

Inoculation and degradation with SSF

A 250-mL Erlenmeyer flask containing 15 g of corncob powder and 45 mL of H_2O was sterilized at 121 °C for 60 min. After the flasks had been cooled, a total of 1×10^6 spores from the strain slants were inoculated aseptically into the flasks. The flasks were incubated at 28 °C for 5 days. This culture was later used as seed to treat corn stalk.

Unsterilized corn stalk were fully mixed with 0% (the control), 1%, 2%, 3%, and 4% H_2O_2 aqueous solution, respectively, and reacted for 12 h at ambient temperature. Then, 15 g of the seed culture medium was inoculated into a plastic dish containing 150 g of H_2O_2-treated corn stalk and mixed thoroughly. The dishes were sealed with a preservation film to maintain the humidity. The whole SSF was conducted at 32 °C for 16 days under the stationary state. Samples were taken at regular intervals to analyze the activity of manganese peroxidase (MnP), lignin peroxidase (LiP), carboxymethyl cellulase (CMCase), and filter paper cellulase (FPase), as well as the contents of lignin and cellulose.

Enzyme extraction

A total of 5 g of the SSF sample was transferred to a 250-mL Erlenmeyer flask, and the MnP and LiP was extracted by adding 100 mL of distilled water to the flask. The suspension was stirred at 150 rpm for 60 min at 28 °C. The recovered enzyme extracts were stored in small volumes (1.5-mL tubes) at -18 °C for further analysis.

Enzyme activity

LiP activity was determined as described by Mitchell and Lonsane (1993); one unit (U) of LiP activity was defined as the amount of enzyme required to oxidize 1 μmole of veratryl alcohol to veratryl aldehyde in 1 min at 30 °C. The activity of MnP was measured by UV-Vis spectroscopy (UV-1801, Lite Electronic Science & Technology Co. Ltd, China) at 240 nm using Mn(II) as a substrate according to Kuwahara *et al*. (1984); one unit of MnP was defined as the amount of enzyme required to oxidize 1 μmole of substrate in 1 min at 25 °C. FPase and CMCase were determined by the methods described by Eveleigh *et al.* (2009) and Ghose (1987), respectively. One unit of FPase and one unit of CMCase were each defined as the amount of enzyme required to release 1 μmole of reducing sugar (expressed as glucose) per min from the original substrate under the experimental conditions.

H_2O_2 hydrolysis

Table 1. Experimental Design on H_2O_2 Hydrolysis of SSF Production for 10 Days

Test number	Production of SSF (g)	H_2O (mL)	H_2O_2 (mL)	H_2O_2 concentration (%)
1	200	600	0	0
2	200	580	20	1
3	200	560	40	2
4	200	540	60	3
5	200	520	80	4

The experimental design used for H_2O_2 hydrolysis during SSF production is shown in Table 1. Two hundred grams of fresh production of SSF for the 10 days using corn stalk treated by 3% H_2O_2 as raw materials, and a special volume of water were loaded into a 3000-mL round-bottom flask with mixing at 100 rpm and preheated in a 37 °C water bath. After the corresponding volume of H_2O_2 was slowly added to the flask in 1 h, hydrolysis was carried out at 37 °C. Samples (8 mL) were removed and concentrated every 3 h to analyze the lignin content.

Chemical analysis

After drying to constant weight, 10 g of sample from the process of fermentation was milled by passing through an 80-mesh sieve. The cellulose and lignin contents of untreated and pretreated corn stalk were determined following National Renewable Energy Laboratory Laboratory Analytical Procedures for standard biomass analysis (NREL LAP) (Sluiter *et al.* 2008) by two-step acid hydrolysis. Lignin is the sum of acid-soluble and acid-insoluble lignin. The acid-insoluble lignin was measured by gravimetric analysis developed by Han and Rowell (1997), and the acid-soluble lignin was measured by UV-Vis spectroscopy (UV-1801, Lite Electronic Science & Technology Co. Ltd, China). All analysis was performed at least in triplicate, and the results are presented as the mean.

RESULTS AND DISCUSSION

Effect of Different Concentrations of H_2O_2 on Ligninolytic Enzymes

The profile of peroxidases synthesized by *A. oryzae* CGMCC5992 under different concentrations of H_2O_2 is shown in Fig. 1. It is evident that when the concentration of H_2O_2 was less than 3%, the activity of both MnP and LiP increased with the increase of the concentration of H_2O_2. Their maximum activities were obtained with 3% H_2O_2.

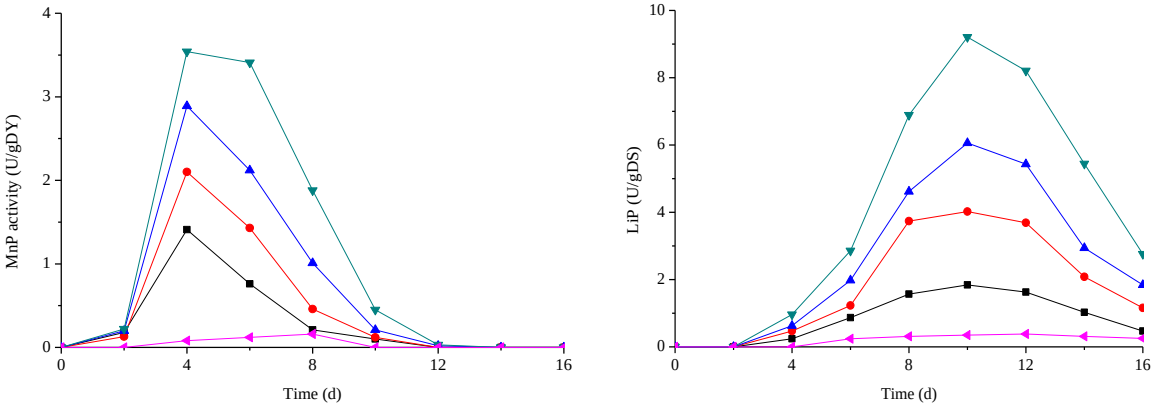


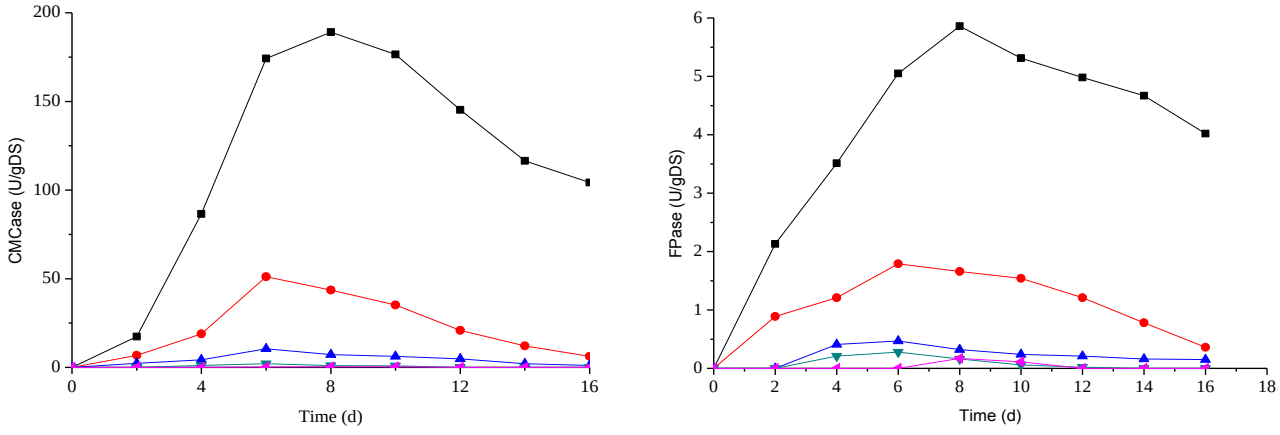
Fig. 1. Variation of ligninolytic peroxidase as a function of H_2O_2 processing time under different concentrations of H_2O_2: (a) MnP; (b) LiP: ■ 0%; ♦ 1%; ▲ 2%; ▼ 3%; and ◄ 4%

The activity of MnP increased from the start to the 4th day, reached its maximum value of 1.88 U/(g dry substrates) in the presence of 3% H_2O_2, and decreased from the 5th day (Fig. 1a). However, the activity of LiP increased from the 4th to the 10th day and reached the peak value 9.21 U/(g dry substrates) under 3% H_2O_2 (Fig. 1b). These data illuminate that H_2O_2 significantly increased the lignin peroxidase activity and facilitated the degradation of lignin in stalk.

Effect of Different Concentrations of H_2O_2 on Cellulase

Cellulose in the corn stalk is the key raw product for synthesizing biofuel. Cellulolytic enzymes hydrolyze cellulose into glucose. To produce more biofuel, cellulose needs to be retained as much as possible in the SSF pretreatment step. Therefore, it is necessary to control cellulose hydrolysis by reducing the amount of cellulolytic enzymes. Cellulose is digested to glucose by the synergistic action of three functional types of cellulolytic enzymes: β-1,4-endoglucanase, β-1,4-cellobiohydrolase, and β-glucosidase (Bayer *et al.* 2004; Lynd *et al.* 2002; Martinez *et al.* 2008). The filter paper cellulase test represents the ability to synergistically hydrolyze cellulose by all three types of enzymes in cellulase, while the CMCase test reflects the activity of β-1,4-endoglucanase.

Both types of enzymatic activity were determined in this study to analyze the effect of H_2O_2 on cellulolytic enzymes. The activity of both CMCase and FPase was reduced with the increase of H_2O_2 concentration (Fig. 2a,b). When the concentration of H_2O_2 reached 4%, both types of enzyme activity were reduced to near zero. The maximum activities of CMCase and FPase were observed in the medium without artificial addition of H_2O_2 at the 8th day as 189.1 U/(g dry substrates) and 5.86 U/(g dry substrates), respectively. These results demonstrated that the activity of cellulase can be turned on and off, controlled by the addition of different concentrations of H_2O_2.

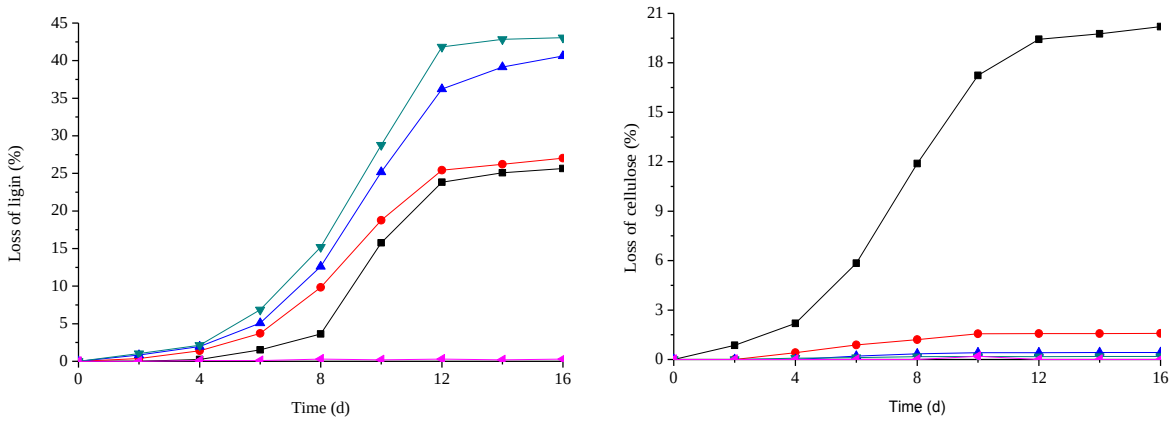


(a) (b)

Fig. 2. Variation of cellulase as a function of H_2O_2 processing time under different concentrations of H_2O_2: (a) CMCase; (b) FPase: ■ 0%; ♦ 1%; ▲ 2%; ▼ 3%; and ◄ 4%

Effect of Different Concentrations of H_2O_2 on the Different Components in Biomass

Plant cell walls, the major reservoir of fixed carbon sources in nature, contain three major polymers: cellulose (30 to 45% w/w), hemicellulose (25 to 45% w/w), and lignin (15 to 30% w/w). The cellulose can be used to produce ethanol after being hydrolyzed by cellulase. However, the lignin in biomass is the major barrier to enzymatic hydrolysis of cellulose. The degradation of lignin after fungal pretreatment can increase the pore sizes in the substrate and provide more accessible surface area to cellulase (Taniguchi *et al.* 2005; Yu *et al.* 2009). To evaluate the effect on lignin degradation in the process of SSF, the loss of lignin and cellulose was determined in this study. As shown in Fig. 3, the lignin degradation was enhanced with the increase of H_2O_2 concentration when the concentration of H_2O_2 was lower than 3%. Lignin digestion occurred from the 4^{th} to the 12^{th} day. The loss of cellulose was evidently reduced in the presence of exogenous H_2O_2 compared with that in the absence of exogenous H_2O_2. With the increase of H_2O_2 concentration, the loss of cellulose was gradually attenuated, indicating that the degradation of cellulose was suppressed (Fig. 3b) and that the activity of cellulase may be on/off controlled.



(a) （b）

Fig. 3. Variation of the major components in biomass as a function of H_2O_2 processing time under different concentrations of H_2O_2: (a) lignin; (b) cellulose: ■ 0%; ♦ 1%; ▲ 2%; ▼ 3%; and ◄ 4%

Hydrolysis of SSF's Production by Its Own LiP

Although lignin was not hydrolyzed after 12 days, the activity of LiP remained high, especially with 3% H_2O_2, suggesting that there is a potential of maximizing chemical removal and degradation of lignin on the 10^{th} day (Fig. 1a). Extensive literature has reported that H_2O_2 is a limited substrate for LiP (Li *et al.* 2010); therefore, it is possible to further degrade lignin in the biomass with artificially supplied H_2O_2. Figure 4 indicates that when the concentration of H_2O_2 was lower than 3%, the lignin digestion in the biomass of SSF was increased with the addition of H_2O_2 during 10 h of hydrolysis. Within 10 h, the loss of lignin increased with time. The maximum total loss of lignin (73.15%) was obtained at 10 h when the biomass of SSF was added with 3% H_2O_2, which was far above the total loss of lignin (41.82%) at the 12^{th} day with 3% H_2O_2 in

conventional SSF. These results further demonstrated that H_2O_2 was a limiting factor in LiP hydrolysis of lignin, and the removal of lignin from the biomass when LiP reached maximum activity during SSF could be further increased by supplementation of H_2O_2 and continuation of the hydrolysis.

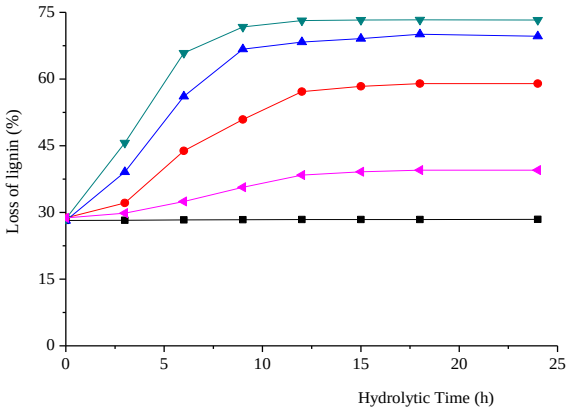


Fig. 4. Variation of the amount of lignin as a function of H_2O_2 hydrolysis time on the 10th day of SSF under different concentrations of H_2O_2: ■ 0%; ♦ 1%; ▲ 2%; ▼ 3%; and ◄ 4%

Lignin in corn stalk is one of the major barriers to saccharifying the cellulose component. The degradation of lignin after fungal pretreatment can increase pore size in the substrate and provide more accessible surface area to cellulase (Taniguchi *et al.* 2005; Yu *et al.* 2009). Versatile peroxidases (such as LiP and MnP) are considered to be crucial enzymes involved in lignin degradation. Hydrogen peroxide is a key oxidizing agent in the degradation process of lignin and other recalcitrant substrates, *i.e.* polyphenols, anthraquinone, *etc.*; it is also widely believed that H_2O_2 from the intermediate metabolism takes part in the reaction of lignin degradation. However, to the best of our knowledge, little research has been focused on the effect of exogenous H_2O_2 on the reaction of lignin hydrolysis by these ligninolytic enzymes. Study of the relationship between different concentrations of exogenous H_2O_2, the synthesis of ligninolytic enzymes, and the hydrolysis reaction of lignin can make full use the function of sterilization and strong oxidation, shorten the time, decrease the cost, avoid environmental pollution, and increase the lignin's removal rate during the pretreatment of corn stalk.

CONCLUSIONS

1. *A. oryzae* grows well in corn stalk pretreated with 3% H_2O_2 without rinsing and does not require additional sterilization.
2. The concentration of H_2O_2 regulates the activities of LiP, MnP, and cellulase synthesized by *A. oryzae* and hence influences the degradation of lignin and cellulose. Therefore, on/off control of the degradation of lignin and cellulose and synthesis of LiP, MnP, and cellulase may be achieved by supplying different concentrations of H_2O_2.

3. The maximum activity of MnP and LiP and minimum activity of cellulase (CMCase and FPase) were obtained with the corn stalk pretreated with 3% H_2O_2. In the process of SSF, the maximum activity of MnP and LiP was obtained with the corn stalk pretreated by 3% H_2O_2 on the 4^{th} and 8^{th} day. On the 8^{th} day, when the biomass in three volumes of H_2O (v/w) was supplemented with 0.25 volumes of H_2O_2 (v/w), the maximum total loss of lignin (73.15%) was achieved in 10 h, which was far higher than that of conventional SSF on the 12^{th} day. Thus the present findings may be applied to the saccharification of stalk.

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