# Lignocellulolytic Enzyme Production in Solid-State Fermentation of Corn Stalk with Ammoniation Pretreatment by *Lentinus edodes* L-8

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This study investigated the effect of ammoniation pretreatment of corn stalk (CS) with different temperatures, ammonia proportions, and processing times on lignocellulolytic enzyme production in solid-state fermentation (SSF) by *Lentinus edodes* L-8. The total N content and lignocellulose contents of ammoniated corn stalk were determined for analysis of the effect of ammoniation pretreatment on lignocellulose structure. The variation patterns of enzyme activity were analyzed according to the enzyme data determined every 2 days during the fermentation. A 4% w/w high-temperature ammoniation pretreatment had a significant effect on cellulase production, and the highest enzyme activity reached almost triple that of the control group. The results also showed that ammoniation pretreatment inhibited the generation of ligninases, such that ligninases appeared later and at lower activities in experimental groups compared to the control group.

*Keywords: Ammoniation; Solid-state fermentation; Ligninase; Cellulase; Lignocellulosic biomass*

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## INTRODUCTION

Corn stalk biomass is rich in lignocellulose, comprising more than 60% of the biomass from corn, with extremely rich production (Tengerdy and Szakacs 2003). China alone produces more than 220 million tons of corn stalk biomass each year. This vast resource is an important renewable resource, usually processed by physical, chemical, or biological methods. By utilization of an underutilized byproduct of agriculture, one can avoid some environmental impacts and pollution associated with some other sources of plant material. The advantages of avoiding pollution, lower cost, and better bioconversion treatment effects make corn stalk a potential source of biofuels, biofertilizers, animal feed, and chemical feedstocks (Grohmann and Himmel 1991; Faheyon*et al.* 1993; Zadrazil and Puniya 1995; Okano *et al*. 2006, 2007). In these applications, the role of cellulases and ligninases is vital because of the saccharification by cellulases and the lignin degradation by ligninases. However, the high cost of cellulase and ligninase production by submerged fermentation (SmF) has limited their application in industry, and efforts have been focused on lower-cost fermentation systems (Pandey *et al*. 1999; Xia and Cen 1999; Thygesen *et al*. 2003; Xu *et al.* 2005; Couto and Sanromán 2005; Kumar and Wyman 2008).

Because of its lower cost, high product concentration, and easy operation, solid-state fermentation (SSF) has been widely studied and applied in antibiotic, enzyme, and

surfactant production (Robinson *et al.* 2001). Especially when compared to SmF, cellulases and ligninases in SSF show higher activities and yield (Couto and Sanromán 2005; Li *et al.* 2006; Sharma and Arora 2010). Enzyme production in SSF often uses white rot fungi; this is because white rot fungi secrete more complete lignocellulolytic enzyme systems. Fermentation substrates used for lignocellulolytic enzyme production by white rot fungi are widely available. In particular, corn stalk rich in lignocellulose is widely used (Tabka *et al*. 2006; Panagiotou *et al.* 2007; Elisashvili *et al*. 2008). Through SSF by white rot fungi, lignocellulose can be completely converted to $CO_2$ and $H_2O$, gaining a fermentation product that can be used as animal feed or fertilizer, achieving the full use of resources (Rodrigues *et al.* 2008). Therefore, selecting white rot fungi with high enzyme-producing capacity is the core of cellulase and ligninase production in SSF. The report of Couto and Sanromán (2005) showed that *Lentinus edodes* would be a kind of fungi producing cellulase and ligninase in SSF.

In addition, the nature of the fermentation substrate is also an important factor affecting the enzyme production of white rot fungi (Couto and Sanromán 2005; Levin *et al*. 2008). Pretreatment with ammonia, *i.e.* ammoniation, can significantly change the physicochemical properties of biomass, damaging the lignocellulose structure and increasing the total N content (Sundstøl and Owen 1984; Liu and Wyman 2005). The damaged lignocellulose structure of corn stalk promotes the cellulose availability for enzyme hydrolysis; and the total N content, especially, the ammonia nitrogen ($NH_3N$) content, plays an important role in meeting the microbes' nutritional requirement for free amino nitrogen and the enzyme production (Taylor *et al.* 2008). Thus, both the damaged lignocellulose structure and the total N content would be focused in the ammoniation pretreatment. The ammoniation pretreatment is greatly influenced by the processing temperature and proportion of ammonia (Sundstøl and Coxworth 1984; Alibes and Kempton 1987). The relationship between processing temperature and processing time was studied, and processing results were determined for other analyses. In this paper, high temperature and normal temperature were applied in the ammoniation pretreatments with different processing times and proportions of ammonia. Treated corn stalk was used as the fermentation substrate in the experimental groups, and the related lignocellulolytic enzyme production by *L. edodes* L-8 was studied.

## EXPERIMENTAL

### Substrate and Organism

Corn stalk (CS), collected from a suburb of Tianjin province, China, was crushed (particle size of about 2±0.5 mm), cleaned with distilled water, and stored at room temperature after drying at 60 °C.

The fungal strain, *L. edodes* L-8, having the capability of degrading lignocellulose and producing lignocellulolytic enzyme, was provided by China General Microbiological Culture Collection Center (CGMCC) and stored in potato dextrose agar (PDA) slants at 4 °C.

### Experimental Design

*Ammoniation of CS*

High-temperature ammoniation (HTA) of CS: different proportions of ammonia were added to CS (in quantities of 3, 4, and 5% w/w of dry matter of CS), and the

moisture content was adjusted to 60%. After thorough mixing, the mixture was placed in a sealed container and heated at 100 °C for different times: 0.5, 1, 1.5, 2, 2.5, and 3 h.

Normal-temperature ammoniation (NTA) of CS: ammonia was added to CS (in a quantity of 4% w/w of dry matter of CS), and the moisture content was adjusted to 60%. After thorough mixing, the mixture was placed in sealed bags at 30 °C.

After the ammoniation pretreatment, the ACS was prepared for chemical analysis.

*SSF of ACS and CS*

The ACS was directly used as SSF substrate after ammoniation pretreatment without other treatments. The experimental groups comprised ACS (average thickness of about 5 to 6 cm) placed in sterilized fermentation trays, to which was added 15% v/w sterile salt solution consisting of (g/L) $KH_2PO_4$, 2 g; $CuSO_4 \cdot 5H_2O$, 0.2 g; $ZnSO_4 \cdot 7H_2O$, 0.05 g; and $MnCl_2 \cdot 4H_2O$, 0.03 g (pH=5.0). The pH of the fermentation substrate was adjusted to 6 with 1 M HCl or 1 M NaOH. The trays were inoculated with 10% *L. edodes* L-8 liquid seeds, and the humidity was adjusted to 60% using sterile water. Subsequently, the fermentation trays were incubated at 27±1 °C and 70% air humidity. The control group included non-ammoniated CS as the substrate, with the rest of the constituents being the same as those used in the experimental groups. Samples of the fermentation substrate were acquired every other day for data measurement.

## Analytical Methods

*Enzyme extraction and assays*

To prepare the enzyme extract in SSF, 1 g of fermented residue was mixed with 20 mL of distilled water. Then, the mixture was shaken at 4 °C and 150 rpm for 2 h, followed by centrifugation (10000 g; 10 min) at 4 °C. The supernatant was obtained for further analysis.

Laccase activity was determined by monitoring the A420 change related to the rate of oxidation of 1 mM 2,2'-azino-bis-[3-ethyl-benzthiazoline-6-sulfonate] (ABTS) in 50 mM sodium acetate buffer (pH 4.2). The enzyme assays were performed in 1-mL cuvettes at 20±1 °C with 50 µL of adequately diluted culture liquid. One unit of enzyme activity was defined as the amount of enzyme that could catalyze the oxidation of 1 µmol of ABTS per minute (Elisaahvili *et al.* 2008). Manganese peroxidase (MnP) activity was measured by oxidation of phenol red (Glenn and Gold 1985). One-milliliter reaction mixtures were incubated for 1 to 5 min at 20 ± 1 °C in the presence of 0.1 mM $H_2O_2$. The reaction was terminated with 50 µL of 4M NaOH, and the absorbance was read at 610 nm. One unit of enzyme activity was expressed as the amount of enzyme required to oxidize 1 µmol of phenol red in 1 min. Activities in the absence of $H_2O_2$ were subtracted from the values obtained in the presence of hydrogen peroxide to establish the true peroxidase activity.

The total cellulase activity (filter paper activity, FPA) was assayed according to IUPAC recommendations using filter paper as the substrate (Ghose 1987). A reaction mixture containing a string of filter paper (Whatman No. 1), 0.5 mL of 50 mM citrate buffer (pH 5.0), and 0.5 mL of appropriately diluted supernatant, was incubated at 50 °C for 60 min. Carboxymethyl cellulase (CMCase) activity was determined by mixing 0.5 mL of appropriately diluted sample with 0.5 mL of 1% CMCase (low viscosity) in 50 mM citrate buffer (pH 5.0) and incubating the mixture at 50 °C for 30 min (Ghose 1987). Reducing sugar was determined by the DNS method, using glucose as the standard curve

(Miller 1959). One unit of enzyme activity was defined as the amount of enzyme that could catalyze the release of 1 µmol of reducing sugars per min.

Enzyme activities were defined as U per g dry substrate (U/g). The analyses were performed in triplicate.

*Total N content and ammonia nitrogen ($NH_3N$) content*

The total N contents of the samples were estimated by the Kjeldahl method according to the AOAC (1990) procedures. The $NH_3N$ contents of the samples were estimated as described by Dryden and Kempton (1983). The $NH_3N$ was extracted by steam distillation of the alkaline solution into boric acid, then analysed by the Kjeldahl method. The analyses were performed in triplicate.

*Estimation of lignin, cellulose, and hemicellulose contents*

The hemicellulose, cellulose, and lignin contents were determined according to Van Soest's method (1991) using a Foss Fibertec 2010 (Sweden) device. The hemicellulose content was estimated as the difference between neutral-detergent fiber (NDF) and acid-detergent fiber (ADF), and the cellulose was estimated as the difference between ADF and acid-detergent lignin (ADL). Lignin was estimated as the difference between ADL and ash content. The analyses were performed in triplicate.

*Determination of biomass*

The fungal biomass in SSF was determined by estimation of N-acetyl glucose-amine according to Roopesh *et al.* (2006), and the biomass was depicted as mg of glucosamine per g dry substrate (mg/g). The analyses were performed in triplicate.

*Scanning electron microscopy*

The ACS and CS samples were dried at 40 °C for 2 h before testing. The scanning electron microscope (SEM) FEI Quanta200 (USA) was used for the observation of the microstructure of the samples. The microscope was operated at a voltage of 15 kV.

*Statistical analysis*

The data processing and statistical analysis were performed by SPSS V22.

## RESULTS AND DISCUSSION

### Effect of Ammoniation Pretreatment of CS

Ammoniation pretreatment can increase the N content of corn stalk, break the lignocellulose structure, and decrease the lignin content. During ammoniation pretreatment, the following ammonolysis reaction between $NH_3$ and the lignocellulose of CS occurred,

$$R–COO–R\bullet + NH_3 \rightarrow R–CO–NH_2 + HOR\bullet \quad (1)$$

where R• denotes a long-chain cellulose and R can indicate polysaccharide chains, a hydrogen atom of hydroxybenzene, or a phenylpropane unit of lignin (Wu *et al*. 2013). The ammonolysis reaction not only improves N content by adding $NH_3$ to lignocellulose, but also breaks down the lignin chain and releases cellulose (Singh *et al*. 2010b).

The total N content, $NH_3N$ content, and the lignocellulose content of HTA and NTA of CS were measured, and the results are shown in Fig. 1 and Table 1. Figure 1 shows the total N content variation curves of HTA and NTA CS at different ammoniation processing times. Moreover, it can be seen that for all ammoniation pretreatments, the total N content increased first and then decreased. The maximum total N contents of all the ammoniation pretreatment groups are shown in Table 1, and the maximum occurred on the 15$^{th}$ day of the 4% NTA group, and 2 h of the 3, 4, and 5% HTA groups, respectively. $NH_3N$ contents of the samples with maximum total N contents are also shown in Table 1. The maximum total N contents increased with the increase of ammonia contents added, and so did the $NH_3N$ contents. NTA pretreatment and HTA pretreatment with the same ammonia proportion (4%) gave similar results of total N contents and $NH_3N$ contents. A reason for the decrease of total N contents shown in Fig. 1 might be the varied moisture, which is a considerable factor affecting the total N content of ammoniation pretreatment (Brown and Adjei 1995).

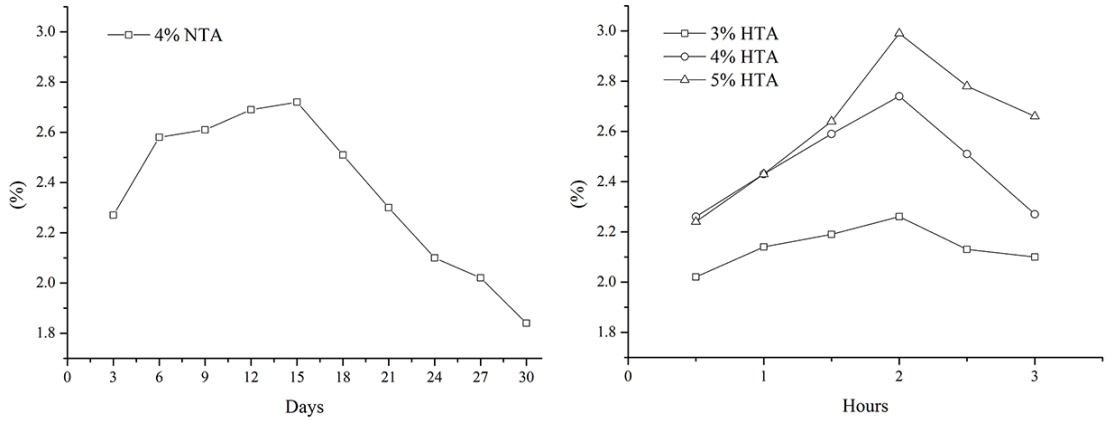


**Fig.1.** Total N content variation curves of HTA and NTA CS at different ammoniation processing times. HTA is high-temperature ammoniation; NTA is normal-temperature ammoniation.

**Table 1.** The Total N Content and the Lignocellulose Content of CS, HTA CS (2h), and NTA CS (15$^{th}$ day) before and after SSF by *L. edodes* L-8

| | Total N % | $NH_3N$% | Lignin % | Cellulose % | Hemicellulose % |
|---|---|---|---|---|---|
| CS | 0.93±0.03 | 0.18±0.02 | 6.25±0.05 | 34.10±0.11 | 27.92±0.04 |
| 4% NTA | 2.74±0.05 | 1.26±0.06 | 5.51±0.04 | 38.58±0.06 | 26.06±0.09 |
| 3% HTA | 2.26±0.06 | 1.02±0.07 | 5.81±0.06 | 36.12±0.04 | 25.88±0.10 |
| 4% HTA | 2.70±0.07 | 1.14±0.13 | 5.43±0.08 | 39.63±0.08 | 25.91±0.13 |
| 5% HTA | 2.99±0.12 | 1.74±0.15 | 5.26±0.06 | 39.77±0.09 | 23.52±0.06 |
| After SSF | | | | | |
| CP | 1.80±0.08 | - | 4.63±0.13 | 31.48±0.05 | 24.73±0.06 |
| 4% NTA | 3.09±0.09 | - | 5.46±0.04 | 32.17±0.08 | 21.16±0.08 |
| 3% HTA | 2.91±0.05 | - | 5.73±0.07 | 31.28±0.09 | 23.26±0.05 |
| 4% HTA | 3.40±0.13 | - | 5.38±0.06 | 30.12±0.12 | 20.37±0.09 |
| 5% HTA | 2.62±0.11 | - | 5.17±0.08 | 37.26±0.07 | 25.38±0.12 |

Data are presented as mean ± SD of 3 replicates.
CP is the control group.

Table 1 shows the lignocellulose content of CS with the maximum total N content of different ammoniation pretreatments. Based on the lignin content with the maximum total N content, the lignin content decreased with increasing ammonia proportion, the significantly greatest ($P<0.001$) decrease in lignin reached 15.8% in 5% HTA CS. With the same ammonia proportion (4%), the lignin decrease of HTA CS was about 1.3% higher than that of NTA CS. In the research of Astuti *et al.* (2011), the lignin content decreased along with the increasing level of urea and the processing time, and the lignin content decreased from 31.79% to 14.749% at the 8% level of urea for two weeks. The hemicellulose content also decreased with increasing ammonia proportions. Degradation of lignin can cause the cellulose content to increase in ACS (Lu *et al*. 2002), which can be found in Table 1. In HTA, with increasing ammonia proportion, the cellulose content increased; maximum cellulose increase reached 16.6% in 5% HTA CS. With the same ammonia proportion (4%), the cellulose increase of HTA CS was 3.1% higher than that of NTA CS.

The similar process results of N contents and lignocellulose contents of HTA and NTA with the same ammonia proportions also show that HTA pretreatment can shorten the processing time. The greater decrease of lignin and hemicelluloses, as well as the corresponding increase in cellulose in the 4% HTA group, in comparison to the 4% NTA group, may be caused by high temperature.

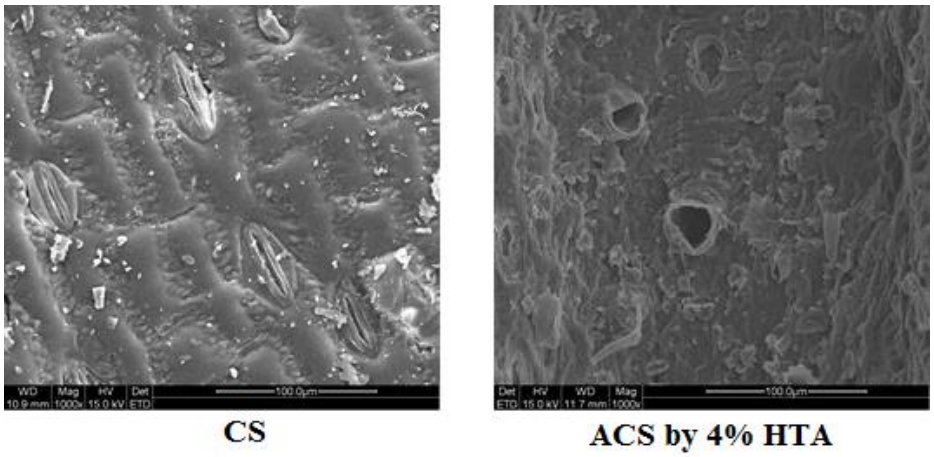


**Fig. 2.** SEM images of the surface of CS and ACS by 4% HTA, taken at magnification 1000X

Figure 2 shows the SEM images of the surface of CS and ACS by 4% HTA. The surface of pretreated fibers was rough, porous, and irregular. Similar results and images were also obtained in earlier studies (Kim and Kim 2012).

These physical and chemical changes of the treated CS increased the surface area of the biomass, which made cellulose more accessible for enzymatic digestion.

Therefore, the fermentation substrates used in the experimental SSF groups were the ACS with the highest total N contents with different ammoniation pretreatments: HTA processed for 2 h at 100 °C and NTA processed for 15 days at 30 °C.

## Effect of Ammoniation Pretreatment on *L. edodes* L-8 Growth

The SSF experiments were carried out for 16 days. Through direct visual and olfactory observation of SSF, the *L. edodes* L-8 mycelium growth rates were faster than that of the control group at the beginning of SSF in all experimental groups, except the 5% HTA group. In all experimental groups, an ammonia smell was detected over the

fermentation trays at the beginning. The smell of 3 and 4% HTA groups and 4% NTA group was weak, but the smell of 5% HTA group was strong. This was likely caused by the highest $NH_3N$ content in 5% HTA group. As SSF time went on, in the 3 and 4% HTA groups and 4% NTA group, the mycelium layer thickened, and the ammonia smell over the fermentation trays gradually weakened and eventually disappeared on about the 8th day. But in the 5% HTA group, the ammonia smell remained strong and the mycelium grew slowly until about the 6th day; and with much of the ammonia volatilized, the ammonia smell gradually weakened and the mycelium grew faster than at the beginning until late in the fermentation.

Although ammoniation pretreatment with highest ammonia proportion can result in highest lignin decrease and highest cellulose increase (Table 1), the highest total N content in ACS can lower the C/N of the fermentation substrate, and inhibit the microbial growth, like the effects in the 5% HTA group. The reason for ammonia smell should also be the $NH_3N$ (residual ammonia) in ACS, as the strongest ammonia smell in 5% HTA group along with significantly ($P<0.001$) highest $NH_3N$ content. But in other experimental groups, $NH_3N$ contents did not inhibit the *L. edodes* L-8 growth. From Fig. 3, 3 and 4% HTA groups and 4% NTA group showed significantly ($P<0.001$) higher growth rates than both of control group and 5% HTA group until 12th day, and achieved significantly ($P<0.001$) higher biomass contents, especially in 4% HTA group and 4% NTA group, showing the promotion of $NH_3N$ contents to fungal growth. After the 12th day when the highest biomass contents were reached in the 4% NTA and HTA group, there was little decrease in the biomass contents, an observation that is consistent with the decrease of cellulases activities shown in Fig. 4.

If evaporation or drying is adopted to reduce the $NH_3N$ content of ACS treated at the high ammonia proportion (5%) to the lower levels, the ACS would also be suitable for microbial growth. But ammonia loss may cause the low fungi growth rate and enzyme yield, as well as increased processing cost when evaporation or drying will be against the purposes of raising the enzyme yield and reducing the enzyme production cost.

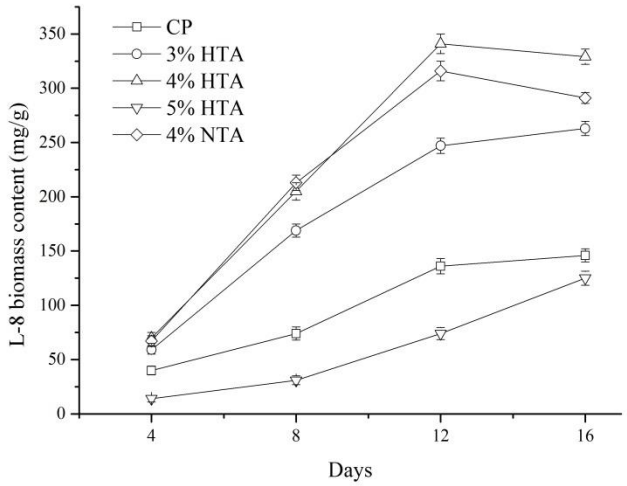


**Fig. 3.** L-8 biomass contents in experimental groups and control group during the SSF. CP (□) is the control group, 3% HTA (○) is the 3% HTA group, 4% HTA (△) is the 4% HTA group, 5% HTA (▽) is the 5% HTA group, and 4% NTA (◇) is the 4% NTA group.

Through the analysis of ACS properties and experimental phenomena of SSF of ACS, it can be found that the ACS with ammoniation pretreatment at the low ammonia proportion, such as 3% or 4%, is suitable as a fermentation substrate for *L. edodes* L-8 growth. Higher *L. edodes* L-8 growth rates and biomass contents, which are the guarantees for higher enzyme activity and yield, also showed the good effect of ammoniation pretreatment.

## Effect of Ammoniation Pretreatment on Enzyme Production by *L. edodes* L-8 in SSF

The laccase and MnP, FPA, and CMCase activities of *L. edodes* L-8 in SSF were determined every other day. Figure 4 shows the laccase, MnP, CMCase, and FPA curves. From Fig. 4, the different variation patterns of the enzyme activities of the experimental groups and control group can be observed. In all experimental groups, except the 5% HTA group, the cellulases appeared first, and then reached high levels on the 6$^{th}$ day, but the ligninases appeared late in the fermentation, with low but increasing activities. As in the report of Singh *et al*. (2011), using rice straw and hulls with microwave–alkali pretreatment as substrate, the maximum CMCase activity (235.6 U/g) was reached on the 6$^{th}$ day of fermentation.

In the control group, however, ligninases appeared first and achieved significantly ($P<0.001$) higher activities on the 12$^{th}$ day, but the cellulases activities were at lower levels until late in the SSF.

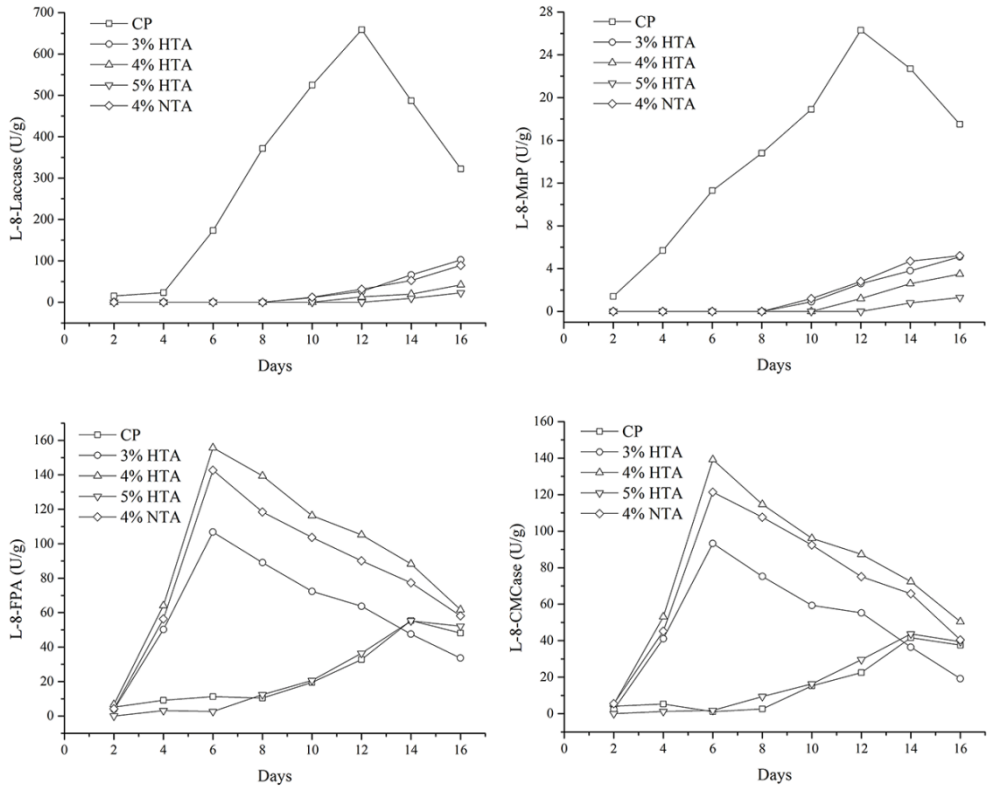


**Fig. 4.** Laccase, MnP, FPA, and CMCase activities of *L. edodes* L-8 during SSF. CP (□) is the control group, 3% HTA (○) is the 3% HTA group, 4% HTA (△) is the 4% HTA group, 5% HTA (▽) is the 5% HTA group, and 4% NTA (◇) is the 4% NTA group.

These two completely opposite enzyme production trends suggest that ammoniation pretreatment has a great influence on cellulases and ligninases produced in SSF by *L. edodes* L-8, acting to significantly promote cellulase production as well as inhibit ligninase production. The promotion of cellulase production can be clearly seen in the experimental groups of 3 and 4% HTA and 4% NTA. The highest CMCase and FPA reached 139.2 U/g and 155.7 U/g, respectively, in the 4% HTA group, almost triple that of the maximum in the control group. The 4% NTA values reached 121.4 U/g and 142.7 U/g, respectively; and the maximum CMCase activity and FPA of all the experimental groups appeared on the 6$^{th}$ day, except the 5% HTA group, 8 days earlier than that of the control group. The promotion of cellulase production was not reflected in 5% HTA because of the inhibition of *L. edodes* L-8 growth described above. The decreasing trends of cellulases activities in experimental groups of 3 and 4% HTA and 4% NTA may be due to the reduction in cellulose contents and nutrients supplied in the substrate, and the accumulation of cellulobiose (Singh *et al.* 2009). The CMCase activity and FPA in the control group stayed at low levels at the beginning of SSF, then quickly increased from about the 8$^{th}$ day, and reached the maximum of 41.6 and 55.4 U/g on day 14, respectively. The research of Narra *et al.* (2012), obtained 10.96 U/g of FPA using rice straw without any pretreatment by solid state fermentation with *Aspergillus terreus*. The results of their study might also show that without pretreatment, using raw biomass as substrate may not obtain high cellulases activities and yields.

The inhibition of ligninase production was also reflected in all the experimental groups, and the inhibition was enhanced with increasing total N content of the fermentation substrate (Commanday and Macy 1985), beginning on the day that laccase and MnP first appeared, the 10$^{th}$ day in the 3% HTA and 4% NTA groups, the 12$^{th}$ day in the 4% HTA group, and the 14$^{th}$ day in the 5% HTA group. But the increasing tendencies of laccase and MnP activities are showed in the Fig. 4, and reaching the maximum at the end of SSF; these values were only about one sixth and one fifth that of the control group, respectively. The control group, using CS as the fermentation substrate produced high laccase and MnP activities; both reached the maximum at 12 days, 658.8 U/g and 26.3 U/g, respectively. And compared to the highest laccase activity (8.2 U/g) in the research of Singh *et al*. (2011), significantly higher maximum laccase activity in this study also proved the ligninase production capability of *L. edodes* L-8.

Ammoniation pretreatment can change the ligninase and cellulase production trends for *L. edodes* L-8 in SSF, and different ammoniation pretreatments showed different effects on enzyme production. In the 3 and 4% HTA and 4% NTA experimental groups, similar cellulase production trends were found: the FPA and CMCase increased until the 6$^{th}$ day, reaching a maximum, then decreased; throughout nearly the entire SSF period, the FPA and CMCase of 4% HTA was the highest, followed successively by 4% NTA, then 3% HTA. There was a small difference in FPA and CMCase between 4% HTA and 4% NTA, which may be due to the more available cellulose caused by high temperatures, and a larger difference existed between 4% and 3% ammoniation pretreatment for both HTA and NTA. Neither a low ammonia proportion (3%) nor a high ammonia proportion (5%) promoted cellulase production as strongly as did the 4% ammonia proportion. Therefore, the best ammoniation pretreatment of CS for cellulase production was judged to be 4% HTA.

## Analysis of Enzyme Production Conditions

Ligninases and cellulases have important significance for *L. edodes* L-8 growth. Ligninases can catalyze the degradation of lignin and destroy the lignocellulose structure of biomass; cellulase can catalyze the hydrolysis of cellulose to obtain glucose. Usually in natural conditions, such as the control group, the poor cellulose availability of non-pretreatment biomass caused by lignin requires fungi to secrete ligninases, first to degrade lignin, improving cellulose availability, and then to secrete cellulases to catalyze the hydrolysis of cellulose, obtaining glucose. Ligninases or cellulases are produced by fungi as the stress responses to different environmental stresses, like in SmF, usually taking nitrogen-limited or carbon-limited approaches to promote cellulase or ligninase production. Therefore, the selection of an appropriate fermentation substrate in SSF provides the environmental stress for *L. edodes* L-8 growth and target enzyme synthesis, which can change its enzyme production trends to obtain different enzyme production results (Singh *et al*. 2010a).

In this study, ammoniation pretreatment was used to change the nature of CS. After ammoniation pretreatment, there were three main significant differences between CS and ACS: more N content, broken lignocellulose structure, and higher cellulose availability in ACS. The three main differences should be the reasons for the different ligninase and cellulase production trends of *L. edodes* L-8 in the experimental groups and the control group. When considering this from the viewpoint of environmental stress and microbe irritability influencing the enzyme production of *L. edodes* L-8, sufficient N sources, less lignin chaining, and more available cellulose in ACS made ligninases less important for *L. edodes* L-8 growth, but more cellulases were required for hydrolysis of cellulose (Aro *et al*. 2001; Montoya *et al*. 2012). Thus, from the enzyme activity results in the experimental groups, at the beginning of SSF, almost no ligninases were found in the fermentation substrate, but cellulase activities appeared and reached the maximum at the $6^{th}$ day. Then, cellulases activities decreased, and ligninases appeared late in the SSF, which could also be explained by the changing environmental stress, in the form of decreased free N and available cellulose absorbed and utilized by *L. edodes* L-8 growth, increasing the demand for ligninases and decreasing that for cellulases (Singh *et al*. 2009).

Therefore, ammoniation treatment is an effective biomass pretreatment method, making the fermentation substrate suitable for cellulase production by *L. edodes* L-8. Furthermore, cellulases produced by ACS SSF with high activities were ligninase-free, making them purer.

But in the control group, the contrary circumstance made *L. edodes* L-8 respond in a different way with regards to ligninases and cellulases production, as described before, showing the possibility of ligninase production by fungi in the method of the control group.

## Analysis of SSF Product Property

Table 1 shows the total N, lignin, cellulose, and hemicellulose contents of the fermentation substrate at the end of SSF. The total N content and cellulose decrease of the experimental groups were significantly higher ($P<0.001$) than those of the control group, except for the 5% HTA group, but the lignin decrease was significantly less ($P<0.001$) than that of the control group. 4% HTA group had the highest total N content increase and cellulose decrease, 0.70% and 24.0%, respectively, followed by the 4% NTA group and 3% HTA group. These results are primarily related to ligninases and

cellulases produced by *L. edodes* L-8 in these groups. In the experimental groups, high cellulase activities increased the cellulose decrease, promoting *L. edodes* L-8 nutrition growth and improving the nutritional content of the fermentation products. In the control group, the high ligninase activities promoted the decrease of lignin, 25.9% compared to 1.7% (the highest lignin decrease in the experimental groups), but lower cellulases activities resulted in slow growth of *L. edodes* L-8 and low nutritional content of the fermented product; the total N content and cellulose decrease in the control group were just 1.80% and 7.7%, respectively.

The results in Table 1 are the data on the 16$^{th}$ day. If the fermentation time were extended to longer than 16 days, according to the increasing tendency of ligninases in the experimental groups discussed and shown in Fig. 4, what could be foreseen is that ligninase activities would further increase. Moreover the degradation of lignin would be improved, while the digestibility of the fermentation product would be further promoted, making the fermentation product more suitable for animal feed (Agosin and Odier 1985; Hadar *et al*. 1993; Basu *et al*. 2002).

Therefore, SSF of ACS by *L. edodes* L-8 not only can obtain high yield cellulases, but can also result in a nutrient-rich fermentation product, which can be used as feed or fertilizer (Mukherjee and Nandi 2004), thereby improving the industrial chain of biomass reuse.

## CONCLUSIONS

1. Through SSF of ACS by *L. edodes* L-8, appropriate ammoniation pretreatment of CS can influence the enzyme production, acting as a stimulant for cellulase and an inhibitor for ligninase. The best ammoniation pretreatment is 4% HTA, leading to the highest cellulase activities in SSF. This fermentation mechanism of cellulase production without ligninases has an important significance in cellulase production by SSF.
2. In the control group, using untreated CS as fermentation substrate, *L. edodes* L-8 showed higher laccase activity and yield in SSF, which could be further studied for laccase production.
3. In addition, the effect of ammoniation pretreatment on enzyme production also affects the fermentation product of ACS SSF by *L. edodes* L-8, which can be further processed as animal feed.
4. Processing temperature can significantly affect the process of ammoniation pretreatment. Moreover, increasing the processing temperature can shorten the processing time, and also result in significantly higher ($P<0.05$) lignin and hemicelluloses decrease, and cellulose increase.

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