Solid-State Fermentation of Ammoniated Corn Straw to Animal Feed by *Pleurotus ostreatus* Pl-5

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The solid-state fermentation (SSF) of ammoniated corn straw (ACS) by *Pleurotus ostreatus* Pl-5 was investigated. The SSF experiments were carried out for 20 d using ACS and corn straw (CS) as the substrates of the experimental group (EP) and control group (CP), respectively. The effects of the ammoniation pretreatment on the CS lignocellulose structure, fungal growth, enzyme production, and components of CS during the SSF process were analyzed. The ammoniation pretreatment effectively degraded the lignin and hemicellulose contents in the CS, by 15.3% and 7.7%, respectively. Thus, the *in vitro* digestibility (IVD) of the EP was higher than for the CP, and even higher than the ligninase activities (laccase: 661 U/g; MnP: 56.8 U/g) found in the CP. The higher cellulase activities (CMCase: 152.3 U/g; FPA: 224.7 U/g) in the EP improved the cellulose degradation, which also promoted the *P. ostreatus* Pl-5 growth, and the high total N content significantly increased the EP fungal biomass and amino acid contents. A shorter processing time and a higher level of nutrients were achieved by the SSF of ACS, which showed its potential for use in animal feed production.

Keywords: Ammoniation; Solid-state fermentation; Ligninase; Cellulase; Animal feed

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INTRODUCTION

Corn straw (CS) is a biomass solid waste with a rich yield that is often used to feed ruminants. However, CS can only be utilized as a low-level feed because of its low digestibility and poor palatability. Ammoniation processing of biomass has been widely used to combat those problems. In China, it has become an important approach in biomass recycling. The ammoniation of CS can increase the total nitrogen (N) content, break the CS lignocellulose structure, and improve the CS digestibility (Sundstøl and Owen 1984; Liu and Wyman 2005). The ammoniation pretreatment is influenced by the processing temperature. A higher processing temperature results in a shorter processing time (Abidin and Kempton 1981; Sundstøl and Coxworth 1984). Thus, in this study, the ammoniation pretreatment occurred at 100 °C. Nevertheless, ammoniation pretreatment cannot effectively improve the nutritional value and total energy of the CS. Therefore, additional biological processing approaches can be helpful to enhance properties of CS used for animal feed, such as digestibility and nutritional composition. These results will also be of use in solving the livestock feed shortage.

In biological treatments, choosing the appropriate microbe and fermentation pattern has a large impact on the bioconversion of ammoniated corn straw (ACS). White-

rot fungi can secrete ligninase and cellulase, which results in lignin degradation and cellulose utilization, respectively. Thus, white-rot fungi can be used to further improve the degradation and utilization of CS lignocellulose, especially during ammoniation processing (Zadrazil and Puniya 1995; Okano *et al.* 2006, 2007). These fungi can absorb nutrients from lignocellulose degradation and convert inorganic N in the ACS to proteinic N, which provides more effective and richer nutrients for animals (Zadražil and Brunnert 1980; Mukherjee and Nandi 2004; Wan and Li 2010). *Pleurotus ostreatus*, which can secrete ligninase and cellulase and does not present safety issues in the fermentation product, has been used previously as a fermentation strain (Reid 1989; Peiji *et al.* 1997; Basu *et al.* 2002; Shrivastava *et al*. 2011).

Many studies have reported that the SSF process is similar to the microbial fermentation system in nature (in particular, the natural growth conditions of filamentous fungus) with many advantages, such as lower cost and higher yields of ligninase and cellulase compared with submerged fermentation, and it is suitable for animal feed production (Moyson and Verachtert 1991; Pandey *et al.* 1999; Mikiashvili *et al.* 2006; Arora and Sharma 2009; Shrivastava *et al.* 2012). Although SSF technology has a long history, the process has only recently been completely examined and broadly used for the production of antibiotics, surfactants, enzymes, and animal feed (Robinson *et al.* 2001; Couto and Sanromán 2005).

Therefore, in the present study, SSF was employed to ferment ACS with *P. ostreatus* Pl-5. Ligninase, cellulose, fungal biomass, and other parameters were studied during the SSF to analyze the fungi fermentation process. Amino acid content, *in vitro* digestibility (IVD), and other parameters were measured to evaluate the digestibility and nutritional content of the fermentation products and determine their viability as animal feed.

EXPERIMENTAL

All experiments were carried out three times, and the values that are shown are the mean values ± the standard deviation (SD).

Substrate and Organism

The CS, collected from a suburb in Tianjin province, China, was crushed (particle size approximately 2 ± 0.5 mm), washed with distilled water, and then stored at room temperature after drying at 60 °C. The fungal strain *P. ostreatus* Pl-5 (number: 5.00345) was provided by the China General Microbiological Culture Collection Center (CGMCC) and stored in potato dextrose agar (PDA) slants at 4 °C.

Experimental Design

Ammoniation of CS

Ammonia (4% w/w of dry matter of CS) was added to the CS, and the moisture content was adjusted to 70% using sterile water. After thorough mixing, the mixture was placed in a sealed container and heated at 100 °C for 2 h. After analysis, the ACS was used as the substrate in the SSF experiments.

Solid-state fermentation of ACS and CS

In the experimental group (EP): (1) The ACS was added to fermentation trays (40 × 30 × 9 cm), and it formed a layer with an average thickness of 4 to 5 cm. (2) To this, a 15% v/w sterile salt solution, consisting of 2 g/L KH_2PO_4, 0.05 g/L $CuSO_4 \cdot 5H_2O$, 0.1g/L $ZnSO_4 \cdot 7H_2O$, and 0.03 g/L $MnCl_2 \cdot 4H_2O$ (pH = 5.0) was added. (3) The pH of the fermentation substrate was adjusted to 6.0 using 1 N HCl. (4) The fermentation substrate in the trays was inoculated with 10% v/v *P. ostreatus* Pl-5 liquid seeds and well mixed; then the humidity of the fermentation substrate was adjusted to 60% using sterile water. (5) Subsequently, the fermentation trays were incubated at 27 ± 1 °C and 60% humidity.

The control group (CP) had non-ammoniated CS as the substrate, and the remainder of the treatment was the same as for the EP.

The SSF experiments were carried out for 20 d and were performed in triplicate. Samples of the fermentation substrates were acquired every other day for data measurement.

Analytical Methods

Ergosterol analysis

The ergosterol content of the fermentation substrate was analyzed as described by Niemenmaa *et al.* (2008). The samples were ground after being frozen with liquid nitrogen, and 0.25 g of the ground mixture was taken for ergosterol and dry weight analyses as described by Niemenmaa *et al.* (2006).

The ergosterol was assayed by the high-performance liquid chromatography (HPLC) method. The weighed samples were suspended in KOH and methanol and then mixed and saponified, as described by Niemenmaa *et al.* (2006). The samples were extracted twice with 2 mL of hexane and ultrasonicated for 10 min with the solvent. The pooled solvent phases were evaporated to a dry residue with N gas. The dry sterol residue was dissolved in 500 µL of methanol and filtered through 0.2-mm Teflon® filters. The samples were analyzed by HPLC (Waters 600E, Massachusetts, USA) using a 5 µm, 250 × 4.6 mm reverse-phase analytical C18 column (Phenomenex Hypersil, California, USA). A mixture of 90% methanol and 10% 2-propanol-hexane (1:1) was used as the eluent. For the standard, the ergosterol (Sigma, St. Louis, MO, USA) was recrystallized from ethanol (99.5%), and the standards (0 to 100 µg/mL) were analyzed in the same way as the samples. The ergosterol contents were expressed as µg per g of dry fermentation substrate. Three parallel samples were analyzed.

Enzyme assays

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

The laccase activity was determined by monitoring the change in the values of A420 (ε_{420} = 3.6× 10^4 $cm^{-1}M^{-1}$) 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) (Elisashvili *et al.* 2008). 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 min. The manganese peroxidase (MnP) activity was measured by the oxidation of phenol red (Glenn and Gold 1985). In this assay, 1 mL 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 4 M NaOH, and the absorbance was read at 610 nm (ε_{610} = 4.46 × 10^4 $cm^{-1}M^{-1}$). One unit of enzyme activity was expressed as the amount of enzyme required to oxidize 1 µmol of phenol red in 1 min. The activities that occurred in the absence of H_2O_2 were subtracted from the activity values obtained in the presence of H_2O_2 to establish the true peroxidase activity.

The total cellulase activity (filter paper activity, FPA) was assayed using filter paper as the substrate (Ghose 1987). A reaction mixture containing a string of filter paper (Whatman No. 1, Cambridge, UK,), 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. The carboxymethylcellulase (CMCase) activity was determined by mixing 0.5 mL of the appropriately diluted sample with 0.5 mL of 1% carboxymethylcellulose (low viscosity) in a 50 mM citrate buffer (pH 5.0) and incubating the mixture at 50 °C for 30 min (Ghose 1987). The reducing sugar content was determined by the dinitrosalicylic acid (DNS) method (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.

The enzyme activities were defined as U per g of dry substrate (U/g).

Determination of total nitrogen content and amino acid content

The total N content of the samples was determined using the Kjeldahl assay according to the Association of Official Analytical Chemists (AOAC) procedures (AOAC 1990). The amino acids were quantified using an automatic amino acid analyzer (L-8900, Hitachi, Tokyo, Japan). The analysis was performed according to the manufacturer's standard protocols. The samples from the 20^{th} d were hydrolyzed for 20 h in an evacuated, sealed ampoule with 6 N HCl at 110 °C and then adjusted to a constant volume of 50 mL. After acid hydrolysis, 5 mL of the supernatant was removed and dried at 65 °C in a water bath using a rotary evaporation method. Then, 5 mL of 0.02 M HCl was added, and the samples were completely washed down and filtered through a 0.22-µM aqueous-phase filter.

Determination of lignin, cellulose, and hemicellulose contents

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

In vitro digestibility

The *in vitro* digestibility (IVD) of the CS, ACS, EP substrate, and CP substrate was estimated according to Akhter *et al.* (1999) with slight modifications and using two-stage digestion, which included treatment with fecal inoculum prepared in artificial saliva, and then with acidified pepsin (Sharma and Arora 2010a,b). The weight loss in dry matter during the processing was expressed as IVD.

Loss in dry matter (LDM)

The loss in dry matter (LDM) was determined using the following equation,

$$\text{LDM (\%)} = \frac{\text{initial dry weight of substrate (g)} - \text{dry weight of testing substrate (g)}}{\text{initial dry weight (g)}} \times 100 \quad (1)$$

Scanning electron microscopy

The samples were dried at 40 °C for 2 h before testing. The scanning electron microscope (SEM) FEI Quanta 200 (Hillsboro, OR, 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 were analyzed by one-way analysis of variance (ANOVA), and performed using SPSS V22 (IBM, New York, USA).

RESULTS AND DISCUSSION

Effect of Ammoniation Pretreatment on CS

Table 1 presents the differences in the lignin, cellulose, and hemicellulose contents between the raw CS and ACS. After the ammoniation pretreatment, the lignocellulose structure was broken, which resulted in a reduction in the lignin and hemicellulose contents (15.3% and 7.7%, respectively), and a significant increase ($P < 0.001$) in the cellulose content (15.4%). Table 1 also shows the differences in the CS after the ammoniation pretreatment. The total N content in the CS significantly increased ($P < 0.001$), from 0.87% to 2.47%, while the IVD slightly increased, from 17.2% to 18.7%.

Table 1. Total N Content, Lignocellulose Content, LDM, and IVD of the Fermentation Substrates of the EP and CP with Different Fermentation Times

	Total N (%)	Lignin (%)	Cellulose (%)	Hemicellulose (%)	LDM (%)	IVD (%)
CS	0.87 ± 0.12	6.41 ± 0.13	34.37 ± 0.21	28.22 ± 0.13	—	17.2 ± 0.3
6 d CP	1.14 ± 0.08	5.93 ± 0.11	33.25 ± 0.14	28.04 ± 0.11	5.2 ± 0.2	18.4 ± 0.2
12 d CP	1.35 ± 0.13	5.31 ± 0.08	32.21 ± 0.17	26.35 ± 0.18	9.3 ± 0.4	21.2 ± 0.5
18 d CP	1.65 ± 0.16	4.92 ± 0.15	30.12 ± 0.24	23.63 ± 0.17	12.4 ± 0.7	25.6 ± 0.7
ACS	2.47 ± 0.16	5.43 ± 0.18	39.66 ± 0.18	26.06 ± 0.16	—	18.7 ± 0.4
6 d EP	2.52 ± 0.13	5.39 ± 0.11	37.11 ± 0.17	24.13 ± 0.21	8.4 ± 0.4	21.6 ± 0.4
12 d EP	2.93 ± 0.21	5.26 ± 0.14	33.47 ± 0.20	21.03 ± 0.17	15.7 ± 0.3	28.3 ± 0.6
18 d EP	3.79 ± 0.14	5.04 ± 0.16	28.39 ± 0.15	19.82 ± 0.13	20.2 ± 0.9	32.4 ± 0.5

Figure 1 shows the SEM images of the CS and ACS. It was seen that the surface of the ACS was smoother compared with the CS, and cellulose swelling in the ACS was also observed.

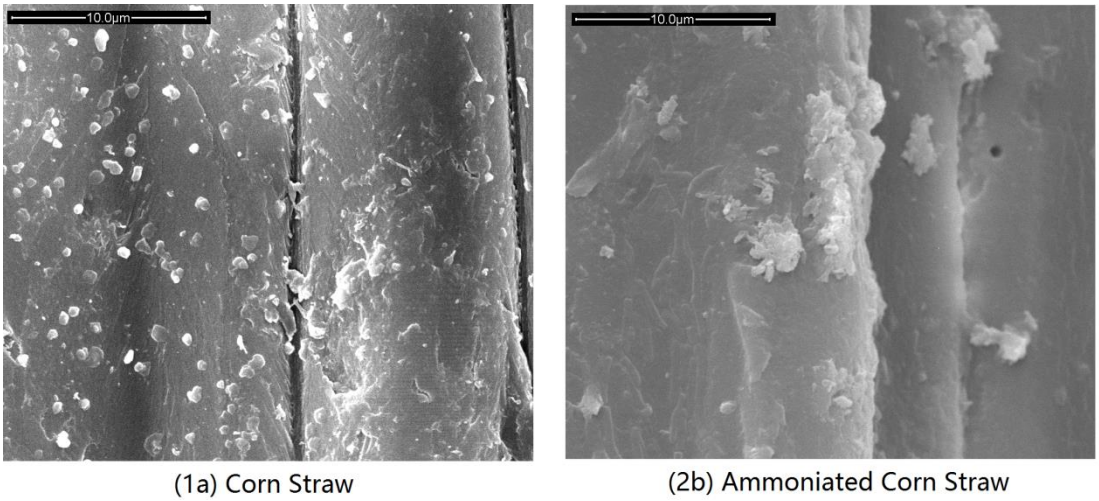

Fig. 1. SEM images of the CS and ACS. The images of the surfaces of the CS and ACS were taken at 10,000X magnification.

The differences between the CS and ACS showed the fine pretreatment results from using high-temperature ammoniation (HTA). One of the reasons for these differences was the ammonolysis reaction between the NH_3 and lignocellulose of the CS. Equation 2 shows the ammonolysis reaction,

$$R–COO–R• + NH_3 \rightarrow R–CO–NH_2 + HOR• \quad (2)$$

where R• indicates the long-chain cellulose, and R indicates the polysaccharide chains, the hydrogen atom of hydroxybenzene, or a phenylpropane unit of lignin (Wu *et al.* 2013). The ammonolysis reaction added NH_3 to the lignocellulose, broke down the lignin chain, and released cellulose, which significantly enhanced the cellulose availability and IVD.

Moreover, the extruding effect of HTA made the ACS more suitable to be used as the fermentation substrate in the SSF process for the fungus. In addition, HTA can effectively kill pests and pathogenic germs in the CS, thus reducing the risk of contamination when scaling-up the SSF process.

The high total N content after the ammoniation pretreatment played an important role in meeting the microbial nutritional and growth requirements for the free amino N (Taylor *et al.* 2008). The changes in the lignocellulose by the ammoniation pretreatment provided better conditions for the enzymatic reactions, which helped the microbes to obtain energy and grow.

P. ostreatus Pl-5 Growth in SSF of EP and CP

Through direct visual observation of the SSF process and the SEM images of the fermentation samples shown in Fig. 2, it was found that more *P. ostreatus* Pl-5 mycelium was present in the EP compared with the CP. In addition, mycelium covered the entire surface of the fermentation substrate in the trays 5 to 6 d after fermentation began. The ergosterol contents of the EP and CP fermentation substrates were determined (Table 2). The ergosterol contents of the EP were significantly higher ($P < 0.001$) than those of the CP. They showed the highest rate of increase during the 8^{th} and 12^{th} d, and reached a maximum on the 20^{th} day. In the CP, the ergosterol content increased more quickly

during the 16th and 20th day than the other fermentation times, and reached a maximum on the 20th day.

Table 2. Ergosterol Contents (μg/g) of the EP and CP Fermentation Substrates during SSF

	4 d	8 d	12 d	16 d	20 d
CP	3.4 ± 1.2	7.3 ± 3.1	11.4 ± 2.3	15.7 ± 1.6	25.6 ± 4.3
EP	9.4 ± 2.5	19.2 ± 4.3	35.4 ± 3.3	42.1 ± 5.6	47.8 ± 3.5

As the primary sterol in the fungal cell membranes and because of its specificity in fungi, the ergosterol content has been widely used as an indicator for the fungal biomass content (Niemenmaa *et al.* 2008). The higher ergosterol contents in the EP indicated that there were higher *P. ostreatus* Pl-5 biomass contents and growth rates than in the CP. As shown in Fig. 2, more *P. ostreatus* Pl-5 mycelium around the CS also suggested a faster fungal growth rate in the EP than in the CP. Thus, the ergosterol contents and direct visual observation supported the conclusion that *P. ostreatus* Pl-5 grew better in the EP than in the CP. Furthermore, the higher holocellulose loss (6 d: 4.52% in EP, and 1.78% in CP) and LDM (6 d: 8.4% in EP, and 5.2% in CP) (Table 1) in the EP also showed that more microbial protein was converted.

The broken lignocellulose structure of the ACS made it more suitable for fungal growth, but the high total N content of the ACS may have had negative effects on fungal growth. Based on the previous analysis, a high total N content of the ACS did not inhibit the growth of *P. ostreatus* Pl-5, but growth was promoted by a sufficient N supply. Thus, as was stated before, the ammoniation process, especially HTA, promoted *P. ostreatus* Pl-5 growth compared to the raw CS.

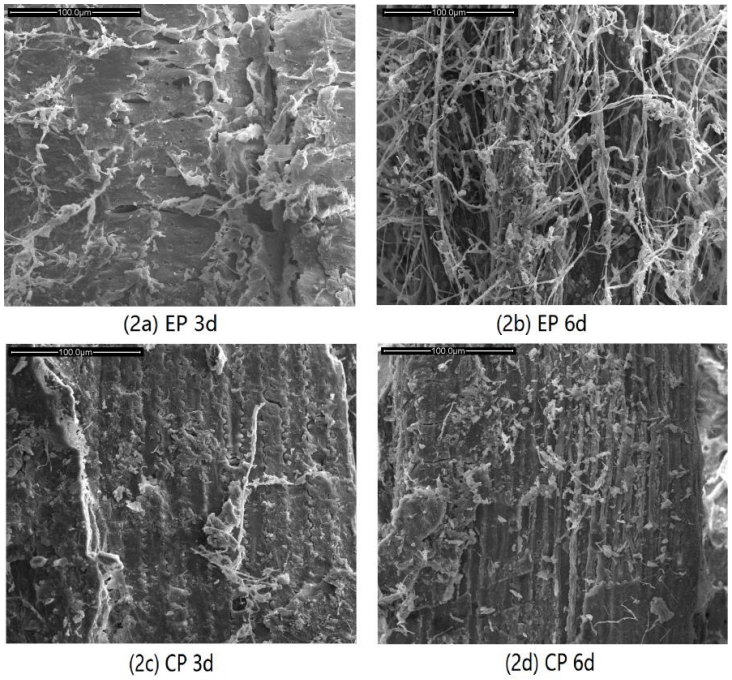

Fig. 2. SEM images of *P. ostreatus* Pl-5 on the CS and ACS fermentation substrates at different fermentation times. The images A and C, and B and D were at the same fermentation times, and taken at 1000X magnification.

Enzyme Production by *P. ostreatus* Pl-5 in SSF of EP and CP

The laccase, MnP, FPA, and CMCase activities of *P. ostreatus* Pl-5 on the substrates were determined every other day. Figure 3 shows the laccase and MnP activity curves, and Fig. 4 shows the FPA and CMCase activity curves of *P. ostreatus* Pl-5. From the figures, the variation patterns and maximum activities of the enzymes in the CP and EP were observed.

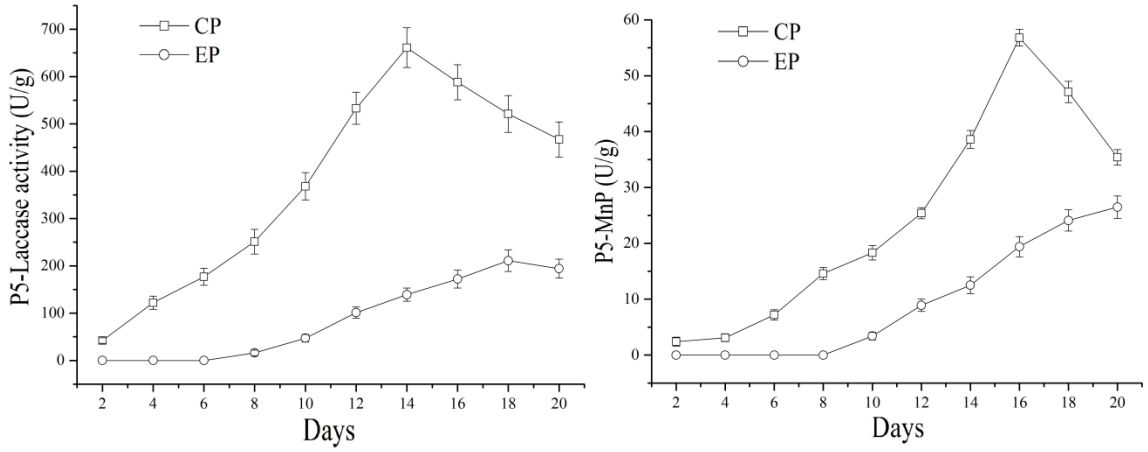

Fig. 3. Laccase activity and MnP activity of *P. ostreatus* Pl-5 during SSF

As shown in Fig. 3, the laccase first appeared on the 8th d, and MnP first appeared on the 10th d for the EP, which was 6 d and 8 d later than for the CP, respectively. The maximum activity of the laccase in the EP reached 221 U/g on the 18th d, which was approximately one-third of the maximum value reached in the CP (661 U/g). The maximum for the EP also occurred 4 d later than for the CP. Similar results were seen for the MnP production. The maximum MnP activity of the EP (26.5 U/g) was approximately half of the CP (56.8 U/g), and appeared 4 d later than in the CP.

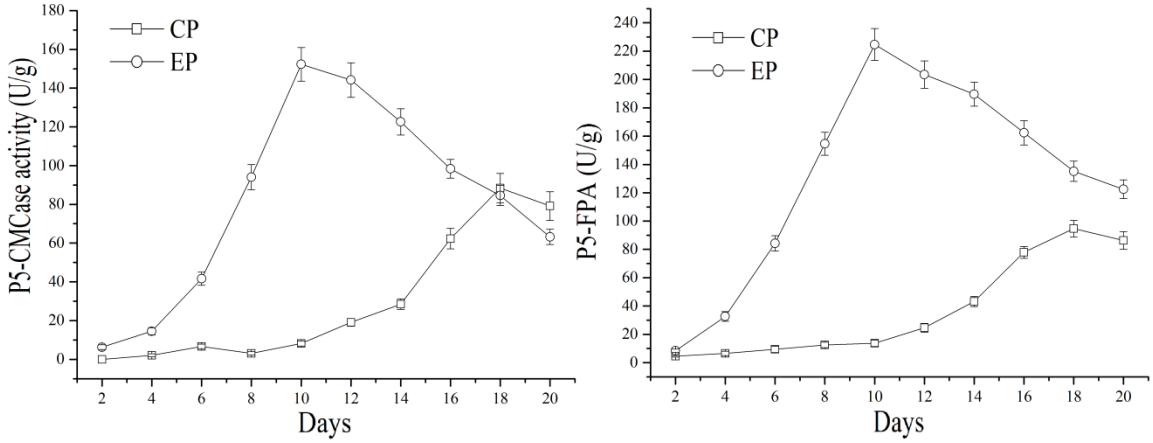

Fig. 4. FPA and CMCase activities of *P. ostreatus* Pl-5 during SSF

The FPA and CMCase activities had similar variation curves, as shown in Fig. 4. In the CP, the CMCase and FPA activities were at very low levels during the early period of fermentation, and increased late in the SSF process. The CMCase and FPA activities reached maximum values of 88.4 and 94.6 U/g on the 18th d, respectively. However, in

the EP, significantly higher ($P < 0.001$) CMCase and FPA activities were evident. The CMCase and FPA activities increased until the maximum values of 152.3 and 224.7 U/g were reached on the 10th d, respectively. These values were almost double the values for the CP and were achieved 8 d earlier than the maximum in the CP. The activities then gradually decreased. The CMCase and FPA activities in the EP remained at high levels during the entire SSF process.

The laccase and MnP were not evident during the early period of SSF of the ACS by *P. ostreatus* Pl-5 in the EP. An excess of N may have been the reason for this. It was reported in earlier studies that an excess of N inhibited the synthesis of ligninase by the fungus (Commanday and Macy 1985; Mikiashvili *et al.* 2006; Arora *et al.* 2011). However, the decrease in the N content caused by the conversion of free N to proteinic N by *P. ostreatus* Pl-5 weakened the inhibition of ligninase. Thus, late in the SSF process, the laccase and MnP activities appeared and increased.

The variable patterns of the CMCase and FPA activities in the CP may have been due to the nature of the fungi that were utilizing the biomass. The early period with low cellulase activity was also the time when the fungal ligninases degraded lignin and enhanced the cellulose availability. The increase in the cellulase activity in later periods represented the need of the fungi to gain energy from the cellulose. However, the ammoniation pretreatment of the CS finished the first step of natural fungal growth, by degrading lignin and enhancing the cellulose availability. Accordingly, in the EP, the period with increasing CMCase and FPA activities occurred earlier, which means that the SSF processing time could be shortened. A shortened SSF processing time is useful for animal feed production. In addition, more available cellulose and N content in the EP may have been the main reasons for the stimulation of cellulase production. More available cellulose was an environmental stress that induced the cellulase production (Aro *et al.* 2001; Montoya *et al.* 2012), and the high N content made it easier for fungal growth and cellulase synthesis.

From the cellulase activities that are shown in Fig. 4 and the ergosterol contents shown in Table 2, it was found that higher cellulase activities accompanied an increase in the ergosterol content in the EP and CP, and also between them. The maximum cellulase activity appeared on the 10th d in the EP and the 18th d in the CP, which was the same period that the ergosterol content increased faster in the EP and the CP, respectively. This suggested that the stimulation of the cellulases by the ammoniation pretreatment promoted the growth of *P. ostreatus* Pl-5. These results showed the importance of cellulases in *P. ostreatus* Pl-5 growth in the SSF process, and also the effects that the ammoniation pretreatment had on the *P. ostreatus* Pl-5 growth.

Lignocellulose Degradation during SSF by *P. ostreatus* Pl-5

During the SSF process, the cellulase and ligninase production directly affected the lignocellulose degradation of the fermentation substrate. Thus, the different patterns of the cellulases and ligninases in the EP and CP resulted in different lignin and cellulose losses, LDM, and IVD. These results are shown in Table 1.

In the EP, the high cellulase activities and low ligninase activities resulted in higher cellulose loss (18th d: 11.27%) and less lignin loss (18th d: 0.39%), respectively. The results in the CP were the reverse. The low cellulase activities and high ligninase activities resulted in less cellulose loss (18th d: 3.08%) and higher lignin loss (18th d: 1.33%), respectively. The LDM of the EP and CP increased as the SSF processing time increased, and the higher LDM in the EP than in the CP at the same fermentation time

was due to the higher cellulose and hemicellulose losses in the EP. The cellulose and hemicellulose losses contributed much more to the LDM than the lignin loss.

Much of the literature agrees that the degradation of lignin could increase the IVD of the CS (Darwish *et al.* 2012). Although the lower ligninase activities in the EP resulted in less lignin loss than in the CP, the final lignin content of the EP was only slightly less than that of the CP because of the effect of the ammoniation pretreatment. Thus, the IVD of the EP was not limited by the lower ligninases and less lignin loss. The IVD of the EP was enhanced from 18.7% to 32.4% by *P. ostreatus* Pl-5, which was higher compared to the increase from 17.2% to 25.6% in the CP. The enhanced IVD and increased LDM in the EP demonstrated that the ammoniation pretreatment effectively promoted the bioconversion of CS by *P. ostreatus* Pl-5. In the report by Sharma and Arora (2011), after 20 d of SSF by *Phlebia floridensis*, the IVD was 25.8% (40% increase in IVD), which was similar to that of the CP in the present study. In the EP, the IVD was 32.4% (73.3% increase in IVD) based on the ammoniation pretreatment and SSF by *P. ostreatus* Pl-5.

Nutritive Quality of Fermentation Products

The crude protein content is an important quality of fermentation products that are used as animal feed. In the present study, the amino acid components of the CS and the fermentation products of the EP and CP of *P. ostreatus* Pl-5 were determined. The results are shown in Table 3. The amino acid profile showed the existential form of N, and high the total amino acid content presented the transformation efficiency of N in ACS by *P. ostreatus* Pl-5. After 20 d of SSF by *P. ostreatus* Pl-5, the total amino acid content increased from 6.46% to 10.83% in the CP, and to 18.82% in the EP. The SSF of the ACS by *P. ostreatus* Pl-5 increased the total amino acid, which were more absorbable and available by animal, improving the nutritive quality compared to that of the CS.

The crude protein content was higher than the total amino acid content because of the presence of other forms of N. The crude protein reached 9.9% with 21 d of SSF of maize stalks by *P. ostreatus* in the report by Darwish *et al.* (2012). The results were similar to that found for the CP, but a significantly higher ($P < 0.001$) crude protein content was achieved in the EP, which showed that the fermentation results were improved by using ACS as the fermentation substrate for *P. ostreatus* Pl-5.

Table 3. Compositional Changes of Amino Acid (g/100 g) after SSF by *P. ostreatus* Pl-5

	Asp	Glu	Gly	Cys	Val	Met	Ile	Ala	Phe
CS	0.34	0.39	0.21	0.46	0.47	0.42	0.49	0.31	0.54
CP	0.74	0.78	0.45	0.59	0.78	0.51	0.62	0.57	0.86
EP	1.32	1.67	0.73	0.74	1.73	0.75	1.55	1.22	1.46
	Thr	Ser	Leu	Tyr	Lys	His	Arg	Pro	Trp
CS	0.08	0.09	0.53	0.51	0.21	0.07	0.79	0.52	0.03
CP	0.32	0.41	0.73	0.77	0.54	0.25	0.91	0.79	0.21
EP	0.41	0.34	1.18	1.37	0.79	0.38	1.34	1.48	0.36

CONCLUSIONS

1. The ammoniation pretreatment degraded lignin, enhanced the cellulose availability, and increased the total N content, as discussed before. It also finished the first step of the natural growth of fungi, shortened the SSF processing time, and achieved a higher nutritive quality.
2. Compared to the non-treated CS, the SSF of ACS by *P. ostreatus* Pl-5 to generate animal feed has good prospects for application because of shorter fermentation times and higher nutrient levels, as shown in this study.
3. Difficulties will be encountered when this process is scaled up. Many factors, such as temperature, pH, moisture, humidity, aeration, and oxygen transfer, may be difficult to control, and the ammoniation pretreatment of biomass also needs further improvement. The large-scale production engineering aspects of the SSF biomass with the ammoniation pretreatment need to be studied.

ACKNOWLEDGMENTS

The authors are grateful for the financial support by the project "International Joint Research Projects in the Science & Technology Pillar Program of Tianjin, China (13RCGFSF14300)", "Research Projects in the Science & Technology Pillar Program of Tianjin, China (14TXGCCX00012)", and the "Fundamental Research Funds for the Central Universities".

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Article submitted: October 23, 2016; Peer review completed: December 12, 2016; Revised version received and accepted: January 7, 2017; Published: January 20, 2017.
DOI: 10.15376/biores.12.1.1723-1736