

# Effect of Fungal Treatments of Rape Straw on Chemical Composition and *in vitro* Rumen Fermentation Characteristics

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The use of rape straw in ruminant production is limited by its high lignin content and low ruminal degradability. White rot fungi are the most efficient known degraders of lignin. Four white rot fungi were investigated for their potential to degrade lignin and improve rumen fermentation of rape straw. Solid state fermentation of the straw was carried out for 0 to 30 days to determine changes in chemical composition and *in vitro* rumen fermentation. Results showed that *Phanerochaete chrysosporium* and *Lentinula edodes* degraded about 45% of lignin and enhanced the *in vitro* organic matter digestibility (IVOMD) and volatile fatty acid production; however, about 55% of the cellulose was lost after 30 days of incubation. *Ceriporiopsis subvermispota* and *Phlebia acerina* degraded a fraction (< 30%) of lignin and cellulose, but inhibited ruminal fermentation. Fungal incubation increased the chitin content of rape straw. Regression analysis showed that the IVOMD increase depended on the combined action of neutral detergent fiber loss and chitin content increase in rape straw. This study indicates that considerations of the conversion of rape straw into ruminant feed with white-rot fungi should take into account the degradation of lignin, fiber loss, and the chitin produced along with the growth of fungi.

*Keywords:* *In vitro* rumen fermentation; Rape straw; White rot fungi

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## INTRODUCTION

Fibrous agricultural by-products as energy substrates for ruminant livestock are staples, but their nutritive feeding values are limited by their low ruminal degradability (Ramirez-Bribiesca *et al.* 2011). Consequently, the majority of these by-products are wasted, either left in the field for natural decay, or burnt, adding to environmental pollution. Rape straw is a good example of a crop residue that is presently not being used efficiently in China. Though almost 7.35 million ha of rape were grown in China in 2011 (China's Rural Statistical Yearbook 2012), little of the rape straw produced was used for animal feed. Rape straw contains considerable quantities of cellulose and hemicellulose and has the potential to be a valuable feed source for ruminants, but the high degree of lignification in the straw particle limits the fermentation of cellulose and hemicellulose by rumen microorganisms (Ramirez-Bribiesca *et al.* 2011). To improve the access of rumen microorganisms to these energy-rich carbohydrates, it is necessary to disrupt the ligno-cellulosic bonds of the plant cell wall (Tripathi *et al.* 2008). Some physical and

chemical methods such as steaming, alkaline, and acidic treatments have been shown to greatly destroy lignin and improve the digestibility of rape straw (Alexander *et al.* 1987). However, the application of these methods presents many disadvantages, including high energy consumption and high risk to the animal and environment, especially when NaOH is used. Biological methods, including the use of white rot fungi and its enzymatic extracts, have the potential to eliminate/reduce the problems associated with physicochemical methods and appear to be the most promising in delignification (Tuyen *et al.* 2012; Arce-Cervantes *et al.* 2013).

White-rot fungi are the most efficient known lignin degraders. White-rot fungi use enzymatic mechanisms to break down lignin, alter lignocellulose structures, and improve the nutritive value of low quality feeds, which has been widely reported using rape straw (Tripathi *et al.* 2008), wheat straw (Tuyen *et al.* 2012), rice straw (Sharma and Arora 2010), corn stover (Tuyen *et al.* 2013), and sugarcane bagasse (Tuyen *et al.* 2013). However, the accompanied losses of cellulose and hemicellulose are often very high during the degradation of these materials using some white rot fungi, which limits their practical use and necessitates looking towards selective ligninolytic organisms (Arora and Sharma 2009). Tuyen *et al.* (2013) found that *Ceriporiopsis subvermispora* and *Lentinula edodes* were highly selective for lignin over cellulose and had a particularly high potential to improve the nutritive value of highly lignified ruminant feeds, such as rice straw, oil palm fronds, and sugarcane bagasse. *Phlebia* species including *P. brevispora*, *P. radiate*, and *P. fascicularia* have also been reported to be highly selective for lignin degradation and possess the potential capability for improvement of digestibility of agricultural by-products (Arora and Sharma 2009; Sharma and Arora 2010). However, very limited data on the effect of these fungi on the chemical composition and degradability of rape straw have been published. *Phlebia acerina* also is a *Phlebia* species for which its effects not only on rape straw but also on other lignocelluloses materials have not been reported. White-rot fungi are very specific and differ in their capabilities of delignification from one source to another (Tripathi *et al.* 2008). Increases in delignification and digestibility by fungi largely depend on the substrate and fungal strain (Agosin *et al.* 1985; Tuyen *et al.* 2013). Therefore, the present study was carried out to investigate the effects of *C. subvermispora*, *L. edodes*, and *P. acerina* on nutrients losses, chemical composition, and rumen fermentation characteristics of rape straw.

## EXPERIMENTAL

### Fungal Strains and Spawn Preparation

*Phanerochaete chrysosporium*, *C. subvermispora*, and *L. edodes* used for this study were obtained from the Agricultural Culture Collection of China (Beijing, China) and *P. acerina* was from the China General Microbiological Culture Collection Center (Beijing, China). These fungi were inoculated on potato dextrose agar (200 g of potato, 20 g of dextrose, 3 g of  $\text{KH}_2\text{PO}_4$ , 1.5 g of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 10 mg of thiamine, and 20 g of bacteriological agar *per* L of water) and incubated at 25 °C until mycelia colonized most of the agar dish surface. One kilogram of wheat grain was cooked for 40 min, washed in tap-water, and drained. The grains were supplemented with 2 g of lime and 8 g of gypsum and mixed manually. Afterwards, 120 g of grain was placed in a 250-mL Erlenmeyer flask and sterilized in an autoclave at 121 °C for 15 min. After cooling, each

flask was inoculated with three agar disks of 6 mm diameter containing mycelium and incubated at 25 °C in full darkness for two weeks.

### Preparation and Cultivation of Substrate

Rape straw used as a substrate was chopped into lengths of 1 to 2 cm and dried at 65 °C. Fifty grams of dried rape straw and 75 mL of water were placed in a 1000-mL Erlenmeyer flask and left overnight for the water to penetrate into the inner structures of the straw. The wet straw was then sterilized in an autoclave at 121 °C for 15 min. The autoclaved containers were cooled to room temperature and the straw was inoculated aseptically with 1.5 g of previously prepared wheat spawn. The containers with inoculated straw were incubated in triplicate along with the control (autoclaved straw with un-inoculated wheat grain) at 25 °C for 0, 10, 20, and 30 days in a biochemistry incubator. When the culture flasks were harvested, 720 mL of sodium acetate buffer (10 mM, pH 5.0) was added to each flask, which were then shaken at 200 rpm for 20 min. The contents of each flask were filtered on a double layer of nylon cloth (400 mesh) and dried at 65 °C. The filtrate obtained was centrifuged at 8000 rpm for 15 min at 4 °C, and the supernatant was used for laccase and manganese peroxidase (MnP) assays. The dried residue was used for various analytical tests.

### Enzyme Assay

Laccase activities were assayed by measuring the oxidation of 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS,  $\epsilon = 36,000 \text{ cm}^{-1} \text{ M}^{-1}$ ) following the method described by Shrivastava *et al.* (2011). The absorbance change was monitored at 420 nm using a spectrophotometer (U-3900; Hitachi, Japan). The assay was performed in a 1.4-mL cuvette at 25 °C, and the enzyme solution was incubated in 1 mL of reaction liquid containing 0.5 mL of 0.3 mM ABTS and 0.5 mL of 50 mM sodium acetate buffer (pH 5.0). One unit of enzyme activity (U) was defined as the amount of enzyme leading to the oxidation of 1  $\mu\text{M}$  of ABTS *per min*.

Manganese peroxidase activity was assayed by the formation of  $\text{Mn}^{3+}$ -malonate complexes (Wariishi *et al.* 1992). Reaction mixtures (1 mL) consisted of 1 mM  $\text{MnSO}_4$  and 0.1  $\mu\text{M}$   $\text{H}_2\text{O}_2$  in 50 mM malonate buffer at pH 4.5, and absorbance was measured at 270 nm ( $\epsilon = 11,590 \text{ cm}^{-1} \text{ M}^{-1}$ ). One unit of enzyme activity was defined as the amount of enzyme that produced 1  $\mu\text{M}$  of product *per min* under the assay conditions.

### In Vitro Ruminant Fermentation Study

Rumen fluid was obtained from three ruminally fistulated beef cattle fed a diet consisting of 700 g/kg rice straw and 300 g/kg concentrates before morning feeding. The rumen liquid collected was filtered through four layers of cheesecloth and mixed (1:2 v/v) with anaerobic buffer (Cone *et al.* 1996). All manipulations were done under continuous flushing with  $\text{CO}_2$ . Fermentation was conducted in 250-mL serum bottles to which 500 mg of rape straw incubated for 30 days and 60 mL of buffered rumen fluid were added. Bottles were closed and incubated in a shaking water bath at 39 °C for 48 h. All samples were incubated in triplicate. A blank (rumen fluid without sample) was incubated in duplicate for correction of residual organic matter (OM) in samples. Fermentation was terminated by placing the bottles at 4 °C, and the residue was filtered using pre-weighed glass crucibles (porosity 1) under vacuum for the determination of *in vitro* OM digestibility (IVOMD). One milliliter of filtrate was preserved by adding 1 mL of

deproteinizing solution (100 g/L metaphosphoric acid and 0.6 g/L crotonic acid) to determine volatile fatty acids (VFA).

### Pepsin-Cellulase Study

The pepsin-cellulase technique was used to estimate the OM digestibility (enzymatic OMD) of fungal incubated rape straw, as well as non-incubated rape straw, according to De Boever *et al.* (1986). Determination was in triplicate for each sample. Dried and ground samples of 300 mg were incubated for 24 h at 40 °C with 30 mL of pepsin solution (0.2% pepsin in 0.1 M hydrochloric acids) in stoppered 50-mL glass crucibles (porosity 1). After incubation was completed, the crucibles were transferred into a water bath at 80 °C for 45 min and filtered. The crucible residue was rinsed with warm water and incubated for 24 h at 40 °C with 30 mL of a cellulase buffer solution. The cellulase buffer mixture consisted of cellulase (Onozuka R-10, Yakult Pharmaceutical Ind. Co. Ltd., Japan) in sodium acetate buffer solution (0.1 M, pH 4.8). The residue after filtration and washing was used for the determination of OM content.

### In Situ Study

Ruminal disappearance kinetics of neutral detergent fiber (NDF) for rape straw uncultured and cultured with *P. chrysosporium* and *L. edodes* were assessed in an *in situ* experiment. Sixteen gram ground samples from each replication of treatments were evenly mixed according to treatment, *i.e.*, 48 g samples per treatment were used for the *in situ* study. The *in situ* experiment was conducted using three ruminally fistulated beef cattle fed a diet consisting of 700 g/kg rice straw and 300 g/kg concentrates. One gram samples each of dried rape straw were weighed into nylon bags (10 × 20 cm; 400 mesh), which were incubated in the rumen of each cow for 4, 8, 16, 24, 36, 48, and 72 h (two replications per incubation time per treatment). Upon removal from the rumen, the bags were washed thoroughly under running cold water for 2 min and then washed in the cold rinse cycle (20 min) of a washing machine. A separate set of two bags per treatment was rinsed without ruminal incubation (0 h). After rinsing, the sample residues were dried to constant weight at 65 °C for determination of residual dry matter (DM). The NDF content in residues was analyzed to estimate fiber degradation.

The percentage of NDF remaining at each incubation time was fitted to the nonlinear regression model,

$$Y = Be^{-K_d(t-L)} + C \quad (1)$$

where  $Y$  is the the percentage of NDF remaining at time  $t$ ,  $B$  is the potentially degradable fraction (%),  $K_d$  is the fractional rate at which  $B$  is degraded (%/h),  $t$  is the incubation time (h),  $L$  is the lag time (h), and  $C$  is the the indigestible fraction (%). The factors  $B$ ,  $K_d$ ,  $L$ , and  $C$  were determined directly by non-linear regression using the NLIN procedure of SAS 9.1 package (SAS Inst. Inc., Cary, NC). The  $A$  fraction defined as the immediately soluble portion was calculated as:

$$A = 100\% - (B + C) \quad (2)$$

Effective digestibility (ED) of NDF was calculated as,

$$ED = A + B \times [K_d / (K_d + K_p)] \quad (3)$$

where  $K_p$  is the the ruminal outflow rate (%/h), which was set arbitrarily at 0.02.

### Chemical Analysis

The samples were analyzed for DM by drying at 135°C in an airflow-type oven for 2 h, for OM by ashing at 550 °C for at least 4 h, and for N using the Kjeldahl procedure. Ash-free NDF, acid detergent fiber (ADF), and acid detergent lignin (ADL) analyses were carried out according to Van Soest *et al.* (1991). The content of hemicellulose was calculated as the difference between NDF and ADF and cellulose as the difference between ADF and ADL. The loss of nutrients due to the fungal incubation was calculated from the difference between the control and the fermented containers and expressed as a percentage of the total nutrient in the control. The content of chitin in samples was estimated according to the glucosamine hydrochloride content of cell wall hydrolysates (Arora and Sharma 2009). The VFA concentrations in the samples were determined by a gas chromatograph (Agilent Technologies 7820A, USA) equipped with a free fatty acid phase capillary column (30 m × 0.25 mm × 0.33 μm, Lanzhou Atech, Lanzhou, China).

### Statistical Analyses

Data for losses of nutrients, laccase activity, and MnP activity were analyzed according to the GLM procedure of SAS 9.1 package with the following model,

$$Y_{ij} = \mu + F_i + P_j + FP_{ij} + \xi_{ij} \quad (4)$$

where  $\mu$  is the common mean;  $F_i$ ,  $P_j$ , and  $FP_{ij}$  are the fixed effects of fungi type, incubation period, and interaction between fungi type and incubation period, respectively; and  $\xi_{ij}$  is the residual error. Chemical composition and data from in vitro fermentation, pepsin-cellulase, and in situ studies were analyzed by GLM procedure with the following model,

$$Y_i = \mu + T_i + \xi_i \quad (5)$$

where  $\mu$  is the common mean;  $T_i$  is the fixed effect of treatment; and  $\xi_i$  is the residual error. Means and standard errors were determined using the LSMEANS and STDERR statement in PROC GLM. Significance was declared at  $P \leq 0.05$ , and trends were discussed at  $P \leq 0.10$ . When a significant effect of treatment was detected, differences among means were tested using Tukey's multiple comparison tests. Pearson's correlation coefficients and multiple linear regressions between an increase of IVOMD (difference between fungal treatment and control) and nutrients losses and nutrients content changes of rape straw were also established using the SAS 9.1 package.

## RESULTS AND DISCUSSION

### Fermentation Losses of Rape Straw after Incubation with Fungi

Data on the fermentation loss of rape straw are presented in Table 1. All fungi caused a net loss in DM, OM, NDF, ADF, and ADL, and consequently in cellulose and hemicellulose of rape straw during fermentation ( $P < 0.001$ ). In general,  $P$ .

*chrysosporium* and *L. edodes* caused the maximum loss in various measures (except for hemicellulose) mentioned above compared with *P. acerina* and *C. subvermispora* during 30 days of incubation ( $P < 0.001$ ). The losses in DM, OM, NDF, ADF, ADL, and cellulose were almost similar between *P. chrysosporium* and *L. edodes* and averaged 40.8%, 41.6%, 52.9%, 52.8%, 45.7%, and 55.4%, respectively, at 30 days of incubation. The losses in various measures except for hemicellulose were not statistically different between *P. acerina* and *C. subvermispora* and were less than 30% after 30 days of incubation. Minimum loss in hemicellulose was caused by *P. acerina* ( $P < 0.001$ ), and no difference was observed in other fungi.

**Table 1.** Fermentation Losses (%) of Rape Straw at 10, 20, and 30 Days Incubation with Different Fungi Compared to a Control

Item	DM	OM	NDF	ADF	ADL	HC	Cellulose
<i>Phlebia acerina</i>							
10	14.5 <sup>de</sup>	15.5 <sup>de</sup>	13.8 <sup>e</sup>	11.9 <sup>ef</sup>	7.4 <sup>e</sup>	19.6 <sup>d</sup>	13.2 <sup>ef</sup>
20	21.1 <sup>c</sup>	22.0 <sup>c</sup>	22.1 <sup>cd</sup>	21.7 <sup>cd</sup>	16.7 <sup>c</sup>	23.4 <sup>cd</sup>	23.2 <sup>cd</sup>
30	20.8 <sup>c</sup>	21.8 <sup>c</sup>	25.0 <sup>c</sup>	24.8 <sup>c</sup>	19.2 <sup>c</sup>	25.5 <sup>cd</sup>	26.5 <sup>bc</sup>
<i>Ceriporiopsis subvermispora</i>							
10	11.2 <sup>e</sup>	12.5 <sup>e</sup>	14.5 <sup>de</sup>	7.2 <sup>f</sup>	5.2 <sup>e</sup>	33.9 <sup>bc</sup>	8.8 <sup>f</sup>
20	16.7 <sup>cde</sup>	17.8 <sup>cde</sup>	21.7 <sup>cde</sup>	17.0 <sup>cde</sup>	12.4 <sup>cde</sup>	35.6 <sup>bc</sup>	18.4 <sup>cdef</sup>
30	16.8 <sup>cde</sup>	17.7 <sup>cde</sup>	24.9 <sup>c</sup>	17.4 <sup>cde</sup>	12.8 <sup>cde</sup>	47.1 <sup>ab</sup>	18.8 <sup>cde</sup>
<i>Phanerochaete chrysosporium</i>							
10	18.1 <sup>cd</sup>	18.5 <sup>cd</sup>	17.6 <sup>cd</sup>	15.4 <sup>def</sup>	15.9 <sup>cd</sup>	24.2 <sup>cd</sup>	15.2 <sup>def</sup>
20	32.1 <sup>b</sup>	33.1 <sup>b</sup>	38.7 <sup>b</sup>	34.2 <sup>b</sup>	28.2 <sup>b</sup>	52.3 <sup>a</sup>	36.1 <sup>b</sup>
30	41.1 <sup>a</sup>	42.1 <sup>a</sup>	53.1 <sup>a</sup>	53.0 <sup>a</sup>	45.4 <sup>a</sup>	53.4 <sup>a</sup>	55.3 <sup>a</sup>
<i>Lentinula edodes</i>							
10	16.8 <sup>cde</sup>	17.2 <sup>cde</sup>	17.0 <sup>cd</sup>	14.3 <sup>def</sup>	9.4 <sup>de</sup>	24.9 <sup>cd</sup>	15.7 <sup>d</sup>
20	33.0 <sup>b</sup>	33.9 <sup>b</sup>	38.4 <sup>b</sup>	35.0 <sup>b</sup>	31.3 <sup>b</sup>	48.4 <sup>ab</sup>	36.1 <sup>b</sup>
30	40.4 <sup>a</sup>	41.1 <sup>a</sup>	52.6 <sup>a</sup>	52.5 <sup>a</sup>	45.9 <sup>a</sup>	50.7 <sup>a</sup>	55.4 <sup>a</sup>
SE	1.15	1.11	1.60	1.71	1.56	2.84	1.93
Fungi	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Period	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
FungixPeriod	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

<sup>a-f</sup>Within a column, means without a common superscript letter differ,  $P \leq 0.05$ ; DM-dry matter; OM-organic matter; NDF- neutral detergent fiber; ADF- acid detergent fiber; ADL-acid detergent lignin; HC-hemicellulose; SE-standard error.

The losses of nutrients in rape straw due to incubation with *P. chrysosporium* have been reported before. Tripathi *et al.* (2008) found that *P. chrysosporium* was effective in degrading lignin of mustard straw and resulted in lignin loss of approximate 40% at 35 days of incubation. The results in the present study were consistent with this report. However, little information could be found on the fermentation losses of rape straw with *P. acerina*, *C. subvermispora*, and *L. edodes*. Fermentation effects of *Phlebia* spp. (*P. brevispora*, *P. fascicularia*, *P. floridensis*, and *P. radiata*) on other lignocelluloses materials have been observed in some studies. Incubation with *Phlebia* spp. degraded 10.6 to 18.0% of lignin and 1.5 to 6.7% of cellulose in paddy straw after an incubation of 30 days (Sharma and Arora 2010). *Phlebia* spp. also caused significant losses in lignin and cellulose of wheat straw, which ranged from 22.1 to 30.5% and from 17.3 to 26.3%, respectively, at an incubation of 30 days (Arora and Sharma 2009). In the present study, *P. acerina* incubation resulted in lignin loss of 19.2% and cellulose of

25.5% in rape straw after an incubation of 30 days, which is within the range of variation reported by previous studies.

*C. subvermispora* and *L. edodes* have been used widely in fermentation incubation of different biomass feedstocks, such as wheat straw, paddy straw, corn stover, and soybean straw (Sharma and Arora 2010; Wan and Li 2011; Tuyen *et al.* 2013), but caused significant difference in losses of lignin and cellulose. For example, after 6 weeks of incubation, *C. subvermispora* and *L. edodes* caused lignin losses of 16.3% and 4.8% for corn stover and 64.1% and 65.2% for paddy straw, respectively (Tuyen *et al.* 2013), which means that fungal incubation shows various degradation activities to lignocelluloses of different feedstocks. In the present study, losses in lignin of rape straw treated with *C. subvermispora* and *L. edodes* reached 12.8% and 55.4%, respectively, after 30 days of incubation.

Lignin is composed of three common phenylpropane structures including p-hydroxyphenyl (H), syringyl (S), and guaiacyl (G) units, which are linked to polysaccharides (hemicelluloses) *via* various covalent bonds (Ghaffar and Fan 2013). Lignin acts as a matrix together with hemicelluloses for the cellulose microfibrils (Ghaffar and Fan 2013). However, the structure of lignin is amorphous and can considerably differ among biomass species. For example, the respective proportions of H, G, and S units in lignins are 5%, 49%, and 46% for wheat straw, but are 4%, 35%, and 61% respectively for mature maize stalks (Ghaffar and Fan 2014). Therefore, the difference in structure of lignin and lignin-carbohydrate complex between rape straw and other biomass feedstocks may result in different degradation extents of lignin and consequent cellulose degradation incubated with the same fungi.

In the present study, hemicelluloses seemed easier to degrade than lignin and cellulose, which is consistent with the results obtained by Tuyen *et al.* (2013). Hemicellulose was more easily attacked than other components in lignocelluloses, which may be due to a lower degree of polymerization than cellulose and multiple endoxylanases produced by white rot fungi during incubation (Isroi *et al.* 2011).

### Laccase and MnP Activities of Fungi During Incubation

Fungi type ( $P < 0.001$ ) and incubation period ( $P < 0.001$ ) significantly affected the laccase activity produced (Fig. 1). The laccase activity was greater for *L. edodes* than for *P. chrysosporium* ( $P = 0.002$ ) and *P. acerina* ( $P = 0.039$ ) on the 10<sup>th</sup> day and for *P. acerina* on the 20<sup>th</sup> day ( $P = 0.006$ ), respectively. There were no differences in laccase activity on the 30<sup>th</sup> day among fungal treatments.

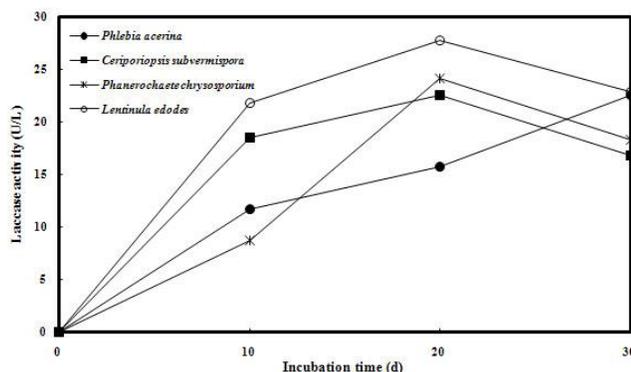
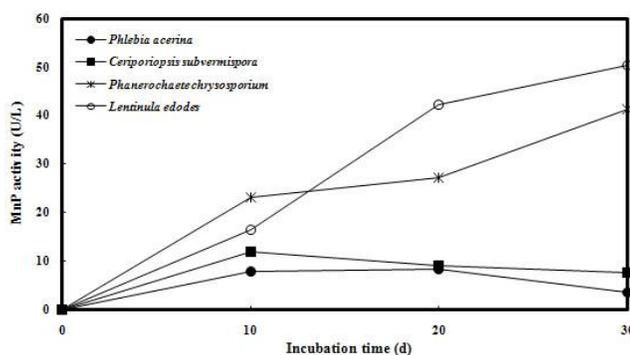


Fig. 1. Laccase activity of fungi during the incubation of 30 days on rape straw

The laccase activity by *P. acerina* increased from 10 to 30 days of incubation ( $P = 0.042$ ). *P. chrysosporium* produced greater laccase activity on the 20<sup>th</sup> day than that on the 10<sup>th</sup> day ( $P < 0.001$ ). Increasing incubation days did not significantly affect the laccase activity by *L. edodes* and *C. subvermispora*.

Manganese peroxidase was affected by fungi type ( $P < 0.001$ ) and incubation period ( $P < 0.001$ ) (Fig. 2). Maximum Mnp activity was shown by *P. chrysosporium* ( $P < 0.05$ ) followed by *L. edodes* on the 10th day of incubation, whose Mnp activity was similar to *C. subvermispora* but greater than *P. acerina* ( $P < 0.001$ ). Maximum Mnp activity was produced by *L. edodes* followed by *P. chrysosporium* on the 20th and 30th days ( $P < 0.001$ ). The Mnp activities in *L. edodes* and *P. chrysosporium* both peaked on the 30th day, but those in *C. subvermispora* and *P. acerina* were not significantly affected by incubation time.



**Fig. 2.** Manganese peroxidase activity of different fungi during the incubation of 30 days on rape straw

White rot fungi use their enzymatic machineries to break down lignin and alter lignocellulose structures. Compared with *P. acerina* and *C. subvermispora*, *L. edodes*, and *P. chrysosporium* produced greater Mnp activity on the 20<sup>th</sup> and 30<sup>th</sup> day in the present study, which was consistent with greater lignin losses observed in the two fungi. Many studies have reported that *L. edodes* and *P. chrysosporium* were capable of producing lignin-degrading enzymes in a variety of lignocellulosic materials. Orth *et al.* (1993) reported that *L. edodes* exhibited higher levels of MnP and laccase activities than other fungi including *P. chrysosporium* on an oak sawdust substrate, and the laccase activity was less than Mnp activity. The current results obtained using rape straw were similar to the cited report.

*C. subvermispora* and *P. acerina* had lower Mnp activity, which may be responsible for less lignin losses in the present study. It was also observed that laccase by *C. subvermispora* and *P. acerina* may play a major role in lignin degradation. The result differed from the production of ligninolytic enzymes in the wood culture of *C. subvermispora*, which indicated that MnP was the main ligninolytic enzyme during the fungal cultivation with less or no laccase detected (Souza-Cruz *et al.* 2004). This discrepancy may be due to the differences in culture conditions. Production of Mnp could be affected by various factors including basal media, temperatures, pH, agricultural residues, and so on (Gill and Arora 2003). Wan and Li (2011) studied the MnP and laccase activities of *C. subvermispora* using different feedstocks and observed that the activity of the enzymes was substrate-dependent; MnP was the predominant ligninolytic enzyme in wood, but in contrast to wood, laccase was the predominant enzyme in

switchgrass. Little information is available on the enzyme production of *P. acerina*. Arora *et al.* (2002) investigated comparatively the difference in ligninolytic ability and associated enzymes between *P. chrysosporium* and *Phlebia* spp. (*P. fascicularia*, *P. floridensis*, and *P. radiate*) and found that all the *Phlebia* spp. tested were capable of producing MnP and laccase. In the present study, the Mnp activity in *P. acerina* was lower but its laccase increased with increased incubation days, which could have contributed to a lignin loss of approximate 19%.

### Changes in Chemical Composition of Rape Straw after Incubation with Fungi

Incubation with fungi increased the ash content of rape straw ( $P < 0.001$ ) (Table 2). The *L. edodes* and *P. chrysosporium* incubations resulted in lower NDF ( $P < 0.001$ ), ADF ( $P < 0.01$ ), and cellulose contents ( $P < 0.01$ ), greater CP content ( $P < 0.05$ ), and similar ADL and hemicelluloses contents compared with the control. Incubation with *C. subvermispora* reduced contents of NDF ( $P = 0.005$ ) and hemicellulose ( $P = 0.044$ ), increased CP content ( $P = 0.033$ ), and did not affect contents of ADF, ADL, and cellulose relative to the control. Incubation with *P. acerina* did not affect NDF, ADF, ADL, hemicelluloses, and cellulose contents, but had the lowest CP content ( $P < 0.01$ ). Incubation with fungi increased the chitin content in rape straw ( $P < 0.001$ ), and *C. subvermispora* produced the maximum chitin content among treatments ( $P < 0.001$ ).

**Table 2.** Chemical Composition (g/100 g DM) of Rape Straw Incubated with Different Fungi for 30 Days

Item	Control	<i>Phlebia acerina</i>	<i>Ceriporiopsis subvermispora</i>	<i>Phanerochaete chrysosporium</i>	<i>Lentinula edodes</i>	SE	p-Value
Ash	3.20 <sup>c</sup>	4.38 <sup>ab</sup>	4.22 <sup>b</sup>	4.95 <sup>a</sup>	4.28 <sup>ab</sup>	0.153	<0.001
NDF	74.3 <sup>a</sup>	71.8 <sup>ab</sup>	68.4 <sup>b</sup>	61.6 <sup>c</sup>	60.9 <sup>c</sup>	0.86	<0.001
ADF	56.4 <sup>a</sup>	54.9 <sup>a</sup>	57.0 <sup>a</sup>	47.4 <sup>b</sup>	46.2 <sup>b</sup>	1.40	<0.001
CP	3.86 <sup>b</sup>	2.84 <sup>c</sup>	4.70 <sup>a</sup>	4.73 <sup>a</sup>	5.04 <sup>a</sup>	0.165	<0.001
ADL	12.2 <sup>ab</sup>	12.4 <sup>ab</sup>	12.8 <sup>a</sup>	11.3 <sup>b</sup>	11.1 <sup>b</sup>	0.32	0.016
HC	18.0 <sup>a</sup>	16.9 <sup>ab</sup>	11.4 <sup>b</sup>	14.2 <sup>ab</sup>	14.7 <sup>ab</sup>	1.37	0.050
Cellulose	40.0 <sup>a</sup>	38.0 <sup>a</sup>	40.0 <sup>a</sup>	31.1 <sup>b</sup>	30.8 <sup>b</sup>	1.22	<0.001
Chitin	0.028 <sup>d</sup>	0.169 <sup>c</sup>	0.305 <sup>a</sup>	0.224 <sup>b</sup>	0.215 <sup>b</sup>	0.0085	<0.001

<sup>a-d</sup>Within a row, means without a common superscript letter differ,  $P \leq 0.05$ ; DM-dry matter; OM-organic matter; NDF-neutral detergent fiber; ADF-acid detergent fiber; ADL-acid detergent lignin; HC-hemicellulose; SE-standard error.

Chemical composition of the substrate was related to losses of nutrients caused by incubation with fungi. In the present study, incubation with *L. edodes*, *C. subvermispora*, and *P. chrysosporium* increased the CP content of rape straw after 30 days of incubation, which was due to a portion of the carbohydrates being degraded, causing a loss of OM. This finding is consistent with the results reported by Tuyen *et al.* (2013). In addition, the increased CP content in the substrates treated with fungi could also be due to capture of N by aerobic fermentation (Abo-Donia *et al.* 2014). However, *P. acerina* reduced the CP content compared with the control in the present study, which contradicted some studies using other agricultural by-products (Tuyen *et al.* 2012, 2013). This disparity may have resulted from the fact that the water-soluble protein or extracellular enzyme protein in liquid fraction secreted by *P. acerina* reduced the CP content in the residual substance. Eriksson and Larsson (1975) pointed out that the intracellular level of protein remains

low if the rot fungi must produce large amounts of extracellular enzyme protein to degrade lignocellulose materials. Hatakka and Pirhonen (1985) have reported that *P. radiate* from the same genus as *P. acerina* was a rather poor producer of mycelial protein, but quite high production of xylanase was observed during solid-state fermentation of wheat straw.

Incubation with *L. edodes* and *P. chrysosporium* caused higher losses in lignocelluloses than in DM, which resulted in lower contents of NDF, ADF, and lignocelluloses in these two treatments. These results were consistent with those of Tuyen *et al.* (2013). On the contrary, incubation with *P. acerina* and *C. subvermispota* did not significantly affect the contents of some components, such as ADF, lignin, and cellulose, which were due to little difference in losses between these components and DM.

As expected, the chitin content in rape straw was increased with the incubation of fungi in the present study. Chitin is a basic structural component of the fungal cell wall. Fungal mycelial growth and infiltration into the substrate thus increases the chitin content (Agosin *et al.* 1985). Fungal chitin content depends upon the species and growing conditions (Arora and Sharma 2009). In the present study, incubation with *C. subvermispota* produced the maximum chitin content in rape straw, which was in agreement with the report by Arora and Sharma (2009), where *C. subvermispota* was richer in chitin content than *Phlebia* spp. Chitin was also detected in the rape straw of the control, which may be because rape straw itself contains small amounts of glucosamine. Martens and Frankenberger (1991) also detected glucosamine from barley straw and alfalfa.

### ***In Vitro* Fermentation and Pepsin-Cellulase Assay of Fungal Incubated Rape Straw**

Compared with the control, greater and lower total VFA concentration was observed in *P. chrysosporium* ( $P = 0.020$ ) and *P. acerina* ( $P = 0.019$ ), respectively (Table 3).

**Table 3.** *In Vitro* Rumen Fermentation Characteristics and Enzymatic OM Digestibility of Rape Straw Incubated with Different Fungi for 30 Days

Item	Control	<i>Phlebia acerina</i>	<i>Ceriporiopsis subvermispota</i>	<i>Phanerochaete chrysosporium</i>	<i>Lentinula edodes</i>	SE	p-Value
Total VFA (mM)	28.3 <sup>b</sup>	21.8 <sup>c</sup>	26.3 <sup>bc</sup>	34.8 <sup>a</sup>	30.9 <sup>ab</sup>	1.18	<0.001
Individual (mM)							
Acetate	19.3 <sup>bc</sup>	15.9 <sup>c</sup>	18.4 <sup>bc</sup>	25.5 <sup>a</sup>	21.5 <sup>b</sup>	0.87	<0.001
Propionate	5.8 <sup>a</sup>	3.0 <sup>b</sup>	3.7 <sup>b</sup>	5.8 <sup>a</sup>	5.7 <sup>a</sup>	0.24	<0.001
Butyrate	2.6	2.2	3.0	2.6	2.6	0.19	0.184
Valerate	0.37	0.29	0.53	0.42	0.59	0.069	0.065
BCVFA	0.24 <sup>c</sup>	0.31 <sup>bc</sup>	0.57 <sup>a</sup>	0.54 <sup>a</sup>	0.52 <sup>ab</sup>	0.046	0.002
A/P	3.33 <sup>d</sup>	5.26 <sup>a</sup>	4.98 <sup>ab</sup>	4.40 <sup>bc</sup>	3.80 <sup>cd</sup>	0.220	<0.001
IVOMD (%)	37.7 <sup>b</sup>	21.7 <sup>d</sup>	33.2 <sup>c</sup>	44.2 <sup>a</sup>	41.2 <sup>ab</sup>	0.81	<0.001
Enzymatic OMD (%)	26.3 <sup>a</sup>	19.3 <sup>b</sup>	21.7 <sup>b</sup>	26.3 <sup>a</sup>	25.9 <sup>a</sup>	0.58	<0.001

<sup>a-d</sup>Within a row, means without a common superscript letter differ,  $P \leq 0.05$ ; OM-organic matter; SE-standard error; VFA-volatile fatty acids; BCVFA-branched-chain VFA; A/P-acetate/propionate; IVOMD-*in vitro* OM digestibility; Enzymatic OMD-enzymatic OM digestibility.

No significant differences in total VFA concentration were found among control, *L. edodes*, and *C. subvermispota* treatments. The maximum acetate concentration was

observed in *P. chrysosporium* among treatments ( $P < 0.001$ ). The propionate concentration was lower for *P. acerina* and *P. chrysosporium* than for other treatments ( $P < 0.001$ ). The control and *P. acerina* treatments had lower BCFA than other fungi treatments ( $P = 0.002$ ). The control and *L. edodes* treatment had relatively lower A/P than other treatments ( $P < 0.001$ ).

*L. edodes* and *P. chrysosporium* treatments had relatively greater IVOMD than other fungi. Though no significant difference in IVOMD was found between control and *L. edodes*, the value in the latter tended to be greater ( $P = 0.08$ ). The minimum IVOMD was observed in *P. acerina* followed by *C. subvermispora* ( $P < 0.001$ ). Enzymatic OMD was lower for *P. acerina* and *C. subvermispora* than for other treatments ( $P < 0.01$ ).

Taking into account higher content of cellulose in the control (40.0%) than in *L. edodes* (30.8%) and *P. chrysosporium* (31.1%), the extent of cellulose degradation may be greater for the latter after digestion of pepsin-cellulase, though enzymatic OMD was similar among these treatments. Treatment of rape straw with *L. edodes* and *P. chrysosporium* resulted in an increase in IVOMD relative to the control during fermentation with mixed ruminal microorganisms, which may be explained by two primary factors. Firstly, incubation with *L. edodes* and *P. chrysosporium* may have changed the cell wall structure in rape straw and increased rumen degradability of NDF or cellulose. In the present *in situ* study, the potential degradability of NDF by *L. edodes* (27.2%) and *P. chrysosporium* (32.0%) were significantly higher than that in the control (19.3%), which resulted in higher ruminal ED of NDF in the two treatments and supported this assumption. Similar to our results, some studies also observed an increase in *in vitro* NDF digestibility, as with IVOMD or IVDMD, from treatment of lignocellulose with different white-rot fungi (Karunanandaa and Varga 1996; Okano *et al.* 2006). Secondly, lower NDF content may have resulted in higher IVOMD in *L. edodes* and *P. chrysosporium* treatments. The NDF content in the control (74.3%) was significantly higher than that in *L. edodes* (60.9%) and *P. chrysosporium* (61.6%), which meant that the latter had higher non-fiber carbohydrates. On average NDF is less digestible than non-fiber carbohydrates. Getachew *et al.* (2004) reported a significantly negative correlation between NDF of feeds and IVDMD.

Unlike *L. edodes* and *P. chrysosporium*, *P. acerina* and *C. subvermispora* both significantly reduced enzymatic OMD and IVOMD of rape straw relative to the control. Inhibition of IVDMD with fungal treatment has also been previously reported, but the mechanism of inhibition is not understood (Jung *et al.* 1992; Karunanandaa and Varga 1996). One possible explanation may be due to the chitin remaining in rape straw after incubation with fungi. Chitin is extremely resistant to degradation by microorganisms (Orpin 1977), and its presence maybe also affected the digestion of other cell wall components. Studies found that the presence of fungal biomass in lignocelluloses reduces adhesion of rumen microbes (Asiegbe *et al.* 1994) and incubation with chitinase increase the *in vitro* DM digestibility of plant residues (Akin 1987). Arora and Sharma (2009) observed that a considerable amount of chitin was included in *C. subvermispora* (320 g/kg), *P. fascicularia* (305 g/kg), *P. radiata* (247 g/kg), and *P. brevispora* (220 g/kg), which limited proportionally the increase of *in vitro* digestibility. In the present study, incubation with *P. acerina* and *C. subvermispora* also increased the chitin content in rape straw, which may have resulted in the reduced IVOMD relative to the control. A second reason may be differences in chemical composition of rape straw. Although white rot fungi can degrade lignin as a source of energy for growth, concurrent degradation of polysaccharide was generally observed. Presumably the polysaccharides removed by

fungal incubation of rape straw represented those polysaccharides with greater ruminal degradability, and considering less losses of lignin in *C. subvermispora* (12.8%) and *P. acerina* (19.2%) treatments, other, more refractory, polysaccharides did not become available.

Production of total VFA during ruminal fermentation is indicative of availability of fermentable energy from the substrate. In the present study, the total VFA concentration closely followed the IVOMD of fungal treated material by ruminal microorganisms, which was consistent with the results by Karunanandaa and Varga (1996). Acetate accounted for about 71% of total VFA and was a primary VFA source; this was because only rape straw used in the ruminal fermentation. In the rumen, BCVFA are derived from branched-chain amino acids (Hristov *et al.* 2004). In the present study, a greater concentration of BCVFA was found in rape straw incubated with *L. edodes*, *C. subvermispora*, and *P. chrysosporium* than in the control, which may be due to lower CP content in the latter. The results were consistent with the report by Hristov *et al.* (2004), where a high ruminally degradable protein diet produced greater BCVFA than a low ruminally degradable protein diet. The CP content was lower in the *P. acerina* treatment than in the control, however, the BCVFA concentration was similar between them, which may be due to the difference in protein composition or branched chain amino acid content. Peiji *et al.* (1997) found that the branched chain amino acid contents in wheat straw and corn straw treated with *Chaetomium cellulolytium* were significantly greater than the corresponding original wheat straw and corn straw. Thus, incubation with *P. acerina* or other fungi may improve amino acid composition or protein quality of rape straw.

#### Ruminal *in situ* Disappearance Kinetics of NDF for Incubated Rape Straw

Incubation with *L. edodes* tended to increase fraction A of rape straw NDF relative to control ( $P = 0.068$ ) (Table 4). The maximum fraction B ( $P = 0.004$ ) and minimum  $K_d$  ( $P = 0.004$ ) was observed in *P. chrysosporium* among treatments. Compared with the control, *L. edodes* and *P. chrysosporium* incubation reduced fraction C by 7.9 and 12.7 percent, respectively ( $P < 0.001$ ). Maximum ED was shown in *L. edodes*, followed by *P. chrysosporium* ( $P < 0.001$ ).

**Table 4.** Ruminal *in situ* Disappearance Kinetics of NDF for Rape Straw Incubated with Different Fungi for 30 Days

Item	Control	<i>Phanerochaete chrysosporium</i>	<i>Lentinula edodes</i>	SE	p-Value
Fraction (%)					
A	4.1	5.5	8.0	0.93	0.068
B	15.2 <sup>b</sup>	26.5 <sup>a</sup>	19.2 <sup>b</sup>	1.45	0.004
C	80.7 <sup>a</sup>	68.0 <sup>c</sup>	72.8 <sup>b</sup>	0.90	<0.001
$K_d$ (%/h)	5.42 <sup>a</sup>	2.13 <sup>b</sup>	5.13 <sup>a</sup>	0.457	0.004
Lag (h)	5.9	6.8	6.0	0.51	0.434
ED (%)	15.2 <sup>c</sup>	19.1 <sup>b</sup>	21.7 <sup>a</sup>	0.44	<0.001

<sup>a-c</sup>Within a row, means without a common superscript letter differ,  $P \leq 0.05$ ; NDF-neutral detergent fiber; SE-standard error; A-the immediately soluble fraction; B-the potentially degradable fraction; C-the indigestible fraction;  $K_d$ -the fractional rate at which B is degraded; Lag-lag time; ED-effective degradability.

To clarify the reason for improved IVOMD in rape straw treated with *L. edodes* and *P. chrysosporium*, an *in situ* degradation study was carried out in the present study.

The results suggested that incubation with fungi increased the ED of rape straw NDF, which explained partially the increased IVOMD observed before. Increased ED for treated rape straw was mainly due to the increased ruminal degradable fraction (A+B), suggesting that fungal treatments loosened the lignocellulosic bonds, thereby facilitating attachment by ruminal microorganisms. Conversely, decreased digestibility of NDF for control might have been due to the cellulose being less digestible in untreated rape straw than when treated, which could be confirmed by greater fraction C in the control. Similar results have been observed in the study by Abo-Donia *et al.* (2014), where incubation with *Trichoderma viride* increased ruminal degradable fraction and ED of peanut hulls DM and OM.

### A Comprehensive Analysis of the Present Study

In summary, incubating rape straw with white rot fungi did not always improve its availability to ruminal microorganisms in the present study, although these fungi all degraded lignin of rape straw. The Pearson's correlation coefficients (*r*) between an increase in IVOMD and losses of OM, NDF, ADF, ADL, hemicelluloses, and cellulose were 0.800 ( $P = 0.002$ ), 0.855 ( $P < 0.001$ ), 0.767 ( $P = 0.004$ ), 0.762 ( $P = 0.004$ ), 0.775 ( $P = 0.003$ ), and 0.764 ( $P = 0.004$ ), respectively. In addition, the correlation between the IVOMD increase and changes in nutrients contents of rape straw was also investigated. The correlation coefficients between the IVOMD increase and the decrease in content of OM, NDF, ADF, ADL, hemicelluloses, cellulose, and the increase in chitin content relative to control were 0.401 ( $P = 0.197$ ), 0.887 ( $P < 0.001$ ), 0.664 ( $P = 0.019$ ), 0.564 ( $P = 0.056$ ), 0.289 ( $P = 0.363$ ), 0.681 ( $P = 0.015$ ), and -0.589 ( $P = 0.044$ ), respectively. The maximum correlation relationship between IVOMD increase and NDF decrease (loss or content) rather than ADL indicated that a change in NDF had the greatest positive effect on the increase of IVOMD, though NDF loss meant loss of partial energy. However, incubation with *P. acerina* and *C. subvermispora* resulted in NDF loss but reduced IVOMD of rape straw, which indicated that other negative factors must be at play, such as chitin produced from fungi. When multiple regression analysis was performed in a stepwise manner with IVOMD increase as dependent variable and nutrients loss and changes of nutrients content as independent variables in the model, a significant equation with a high goodness of fit was obtained (IVOMD increase (%) =  $0.487 \times \text{NDF loss (\%)} - 80.727 \times \text{chitin content increase (\%)} - 5.493$ ;  $R^2 = 0.942$ ;  $P < 0.001$ ). The equation strongly suggested that the increase in IVOMD depended on the combined action of NDF loss and chitin content in rape straw. The result indicated that treating rape straw with fungi results in residual chitin that accompanies the fungi growth. Lignin and other components were finally excluded from the model, which reaffirmed that NDF may have had a greater effect on IVOMD than lignin.

### CONCLUSIONS

1. *L. edodes* and *P. chrysosporium* showed great ability to degrade lignin of rape straw, increase ruminal degradable fraction of neutral detergent fiber (NDF), and improve ruminal fermentation, but resulted in the loss of cellulose to a great extent.
2. *P. acerina* and *C. subvermispora* inhibited ruminal fermentation of rape straw, though a portion of the lignin and cellulose was degraded during incubation.

3. The IVOMD increase depended on the combined action of NDF loss and chitin content increase in rape straw. The chitin produced along with the growth of the fungi should be considered in the conversion of rape straw into ruminant feed with white rot fungi.

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