

# Comparative ligninolytic potential of *Phlebia* species and their role in improvement of *in vitro* digestibility of wheat straw

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## ABSTRACT

Digestibility of crop-residues can be enhanced by the delignification, which improves its nutritive quality. Presence of lignin in these crop-residues which act as digestibility barrier can be degraded by fungi. The biodelignification ability of three *Phlebia* species: *Phlebia radiata*, *Phlebia fascicularia* and *Phlebia brevispora*, was studied under solid state fermentation of wheat straw for 30 days and compared with that of much studied white rot fungus *Ceriporiopsis subvermispora*. Different parameters including laccase activity, loss in total organic matter, water soluble content, hemicellulose, cellulose, lignin, ash content, change in pH and effect of the chitin content of fungus on *in vitro* digestibility were examined before and after the fungal treatment of substrate. *P. brevispora* caused maximum lignin loss of 305 g/kg with the lowest loss in total organic matter (105 g/kg) and enhanced the digestibility to its maximum extent (259 g/kg). *C. subvermispora* which degraded 230 g/kg lignin gave maximum *in vitro* digestibility of 221 g/kg only.

**KEY WORDS:** *in vitro* digestibility, chitin, ligninolytic activity, solid-state fermentation, white rot fungi, wheat straw

## INTRODUCTION

Several crop-residues like wheat straw, rice straw, maize stover etc., are generally used as substitutive fodder as well as its supplement. These residues can be converted into high quality feed by means of fungal delignification, which

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improves its nutritive value (Zadrazil and Brunnert, 1980; Reid, 1989; Vills-Boas et al., 2002). Natural degradation of plant residue is mainly caused by different fungal and bacterial species. Holocellulose and lignin are the biopolymers that are converted into low molecular weight compounds by the enzymatic action of these microorganisms. Fungi are usually better degraders of plant cell wall constituents because of their hyphal penetration power. Many fungal species have been screened for their potential to degrade cereal residues. White rot fungi are the most studied and degrade lignin efficiently by using their ligninolytic enzyme system (Agosin et al., 1985; Arora et al., 2002). Lignin is bound to cellulose and hemicellulose and makes these components inaccessible for microbial attack. Animal enzymatic system does not support ligninolysis and so these crop-residues are not completely digestible and thus of poor nutritive value. Fungal ligninolysis and break down of cellulose-hemicellulose matrix liberate simple degradable components that can be easily utilized by rumen microflora, thus improving the ruminant digestibility.

*Phanerocheate chrysosporium* is a well-known white rot fungus and most widely studied for lignin degradation. However, loss of total organic matter is very high during the degradation of lignocellulosics, which limits its practical use and necessitates looking towards selective ligninolytic organisms (Jung et al., 1992). *Phlebia* species are known selective lignin degraders (Arora and Gill, 2000) and thus may possess the potential capability for improvement of digestibility of agro-residues. Four white rot fungi including three different species of *Phlebia* and another widely studied fungus *Ceriporiopsis subvermispota* (Akin et al., 1995) were selected to find out their comparative role in selective ligninolysis and *in vitro* digestibility of wheat straw. *In vitro* digestibility was measured by using buffalo faecal matter rather than ruminant fluid as a substitutive source of microflora. This is relatively easy method than Tilley and Terry's (1963) two-stage *in vitro* procedure, which requires special surgical skills, fistulated animal, where special care is needed to keep fistula free from any infection. On the other hand, the method involving the use of enzymes, like cellulase, is relatively expensive (Nousiainen et al., 2003). Use of faecal inoculum for determining the digestibility is comparatively cheaper and easier method for practical use in a microbiological laboratory (Shaer et al., 1987; Akhter et al., 1999).

## MATERIAL AND METHODS

### *Organisms*

Four white rot fungi *Phlebia radiata* (MJL-1198), *Phlebia brevispora* (HHB-7030), *Phlebia fascicularia* (FP-70880) and *Ceriporiopsis subvermispota* were

used in the present study. All *Phlebia* spp. were received from T.W. Jeffries (Forest Product Laboratories, Madison, USA). The cultures were maintained by regular subculturing on yeast extract glucose agar (YGA) slants and stored at 4°C as well as at -80°C in 10% (v/v) glycerol.

### *Experimental procedure*

Wheat straw (WS) used as a substrate was milled (particle size 2 mm ± 0.5), washed (with water at 25-30°C) and dried at 90°C. Five g of dried WS taken in 250 ml conical flasks was moistened with 25 ml of 0.5% (w/v) malt extract and after sterilization at 1.06 kg/cm<sup>2</sup> for 15 min, the flasks were inoculated with three mycelial discs (8 mm) per flask, grown on YGA plates for 6 days. All the flasks were incubated at 25°C. Two flasks for each organism were processed at 10, 20, and 30 days along with 2 uninoculated control flasks. The whole experiment was repeated three times. Thus, the data presented in Tables 1 and 2 is an average of six individual flasks. Enzyme extraction was done as described earlier (Arora et al., 2002). Twenty five ml of sodium acetate buffer (10 mM, pH 5) was added to each flask and put on a rotary shaker at 200 rpm for 20 min. The contents of each flask were filtered on a tared filter paper and dried at 90°C until constant weight. Filtrate obtained was centrifuged at 8000 g at 4°C for 15 min and the extract thus obtained was used for laccase assay. The dried residue was used for different analytical tests.

### *Analytical methods*

The sequential fractionation of lignocellulosics was carried out according to Datta (1981) with slight modifications. One g of WS was suspended in 100 ml distilled water, kept at 100°C for 2 h in a water bath and filtered on a tare crucible, residue was dried at 90°C till constant weight. Loss was considered as water soluble part. Dried residue was suspended in 100 ml of 0.5 M H<sub>2</sub>SO<sub>4</sub> and after keeping for 2 h at 100°C in a water bath, the contents were filtered, dried and weighed as described in the first step and loss in weight is represented as hemicellulose content. For cellulose and lignin estimations, 10 ml of 72% (v/v) H<sub>2</sub>SO<sub>4</sub> was added to the above dried residue and kept at 30°C for 1 h on a rotary shaker at 200 rpm. After incubation the mixture was diluted up to 4% (v/v) of H<sub>2</sub>SO<sub>4</sub> and autoclaved at 1.06 kg/cm<sup>2</sup> for 40 min. The contents were filtered, dried and weighed. The loss in weight was treated as cellulose, and the left over residue was considered as lignin.

For estimating the residual ash content, 1 g of sample was kept at 550°C for 5 h in a tare crucible and reweighed to calculate the residual ash content. Change in pH of the decomposed and undecomposed WS was determined by taking

500 mg of WS in 10 ml of distilled water and kept on a rotary shaker at 200 rpm for 30 min. pH was read with the help of pH meter.

#### *Laccase assay*

Laccase (EC 1.10.3.2) activity was measured as described earlier (Arora and Gill, 2005). Five ml of reaction mixture containing 10 mM sodium acetate buffer (pH 5), 2 mM guaiacol and 0.2 ml of culture supernatant was incubated at 25°C for 2 h and the absorbance was read at 450 nm. The relative enzyme activity has been expressed as colorimetric units/ml (CU/ml).

#### *Chitin estimation*

Chitin was measured according to Chen and Johnson (1983). All the fungi were grown in 50 ml yeast extract glucose broth for 15 days at 25°C. The fungal biomass so obtained was dried at 90°C for 24 h. Ten mg of dried fungal biomass was refluxed at 100°C for 4 h in 5 ml of 6 M hydrochloric acid. After cooling to room temperature, hydrolysates were filtered and 1 ml aliquots of such samples were withdrawn and evaporated to dryness at 60°C under reduced pressure. The dry hydrolysates were then redissolved in 4 ml distilled water. To 1 ml of this diluted solution, 0.25 ml of acetylacetone (4% acetylacetone in 2.5 M sodium carbonate) was added and heated at 90°C for 1 h in a capped test tube. After cooling, 2 ml of ethanol and then 0.25 ml of Ehrlich reagent (1.6 g of N-N-dimethyl-p-aminobenzaldehyde in a 30:30 ml mixture of ethanol and concentrated HCl) was added. The colour formed was measured at 530 nm. The glucosamine hydrochloride content of cell wall hydrolysates was determined by comparing its absorbance with that of standard glucosamine hydrochloride.

#### *In vitro digestibility*

*In vitro* digestibility of uninoculated and fungal treated WS was estimated according to Akhter et al. (1999), with slight modifications. Faecal inoculum was prepared by mixing fresh faecal matter of buffalo (100 g/l) in pre-warmed (39°C) artificial saliva (NaHCO<sub>3</sub> 9.80 g, Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O 7.00 g, KCl 0.57 g, NaCl 0.47 g, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.12 g and 1 ml CaCl<sub>2</sub> (4%, w/v) in 1000 ml of distilled water) and filtered through four-layered muslin cloth. Five hundred mg WS (washed and dried) taken in 50 ml centrifuge tube was suspended in 35 ml of faecal inoculum. After flushing with CO<sub>2</sub> gas, these tubes were kept at 39°C for 48 h in a water bath. Supernatant was discarded and 35 ml of acidified pepsin (6.6 g in 1 l of 0.1 M HCl) was added to the residue. Tubes were again incubated at the same conditions for 48 h. Residue was filtered on a tared filter paper and

dried. The weight loss in dry matter during the incubation has been expressed as *in vitro* digestibility.

### *Statistical analysis*

The data was represented as mean with standard deviation and analysed by two-way ANOVA (4 X 3 factorial design) and correlation.

## RESULTS

Solid-state fermentation of wheat straw was carried out with different white rot fungi and compared with that of the properties of uninoculated WS. Uninoculated WS contains 85 g/kg water solubles, 353 g/kg hemicellulose, 325 g/kg cellulose, 240 g/kg lignin and 70 g/kg residual ash with the *in vitro* digestibility of 143 g/kg and pH 6.04. The fungi were examined for their potential to degrade different plant cell wall components and enhancement in *in vitro* digestibility during 30 days of incubation as stationary cultures at 25°C (Table 1).

All the fungi caused variable loss in total organic matter (TOM) during solid state fermentation of WS. *P. radiata* caused the maximum loss (159 g/kg) during 30 days of incubation. *P. fascicularia*, *P. brevispora* and *C. subvermispora* did not show any statistically significant difference in the resultant loss in TOM, which ranged from 100-108 g/kg (Table 1). Different fungi were consistent in their rate of degradation during 30 days period except *P. fascicularia*, which caused more loss during 10-20 days of incubation.

A maximum of 127 g/kg water soluble components were liberated by *C. subvermispora* during 20 days while *P. brevispora* and *P. fascicularia* liberated maximum water solubles during 30 days (120 and 98 g/kg, respectively) and *P. radiata* (97 g/kg) during 10 days. *C. subvermispora* was more effective in the early period of incubation and released more water solubles during first 20 days with very little increase during further incubation while *Phlebia* species were more active in releasing water solubles in 20-30 days period (Table 1).

Hemicellulose was best degraded (200 g/kg) by *C. subvermispora* during 30 days of incubation and followed by *P. fascicularia* (189 g/kg), *P. radiata* (150 g/kg) and *P. brevispora* (118 g/kg) (Table 1). *P. fascicularia* degraded hemicellulose more efficiently during 20-30 days, while all other fungi degraded maximum amount of hemicellulose during initial stages of degradation, i.e. first 10 days of incubation.

*P. fascicularia* was the best degrader of cellulose and caused a maximum loss of 263 g/kg, followed by *P. brevispora* and *C. subvermispora* by causing around 190 g/kg loss, whereas *P. radiata* caused a loss of 173 g/kg in 30 days. *P. radiata*, *P. brevispora* and *C. subvermispora* were more active during 10-20

Table 1. Solid state fermentation of wheat straw and changes in its biochemical composition and digestibility by four different fungi

Item	<i>P. radiata</i>			<i>P. brevispora</i>			<i>P. fascicularia</i>			<i>C. subvermispora</i>			SE	A	B
	Days of incubation									10	20	30			
	10	20	30	10	20	30	10	20	30						
Loss in TOM <sup>1</sup>	38 <sup>b</sup>	81 <sup>c</sup>	159 <sup>e</sup>	33 <sup>b</sup>	61 <sup>c</sup>	105 <sup>d</sup>	12 <sup>a</sup>	65 <sup>c</sup>	108 <sup>d</sup>	37 <sup>b</sup>	73 <sup>c</sup>	108 <sup>d</sup>	11.9	NS	***
Water soluble conc. <sup>1</sup>	97 <sup>c</sup>	68 <sup>a</sup>	94 <sup>c</sup>	82 <sup>b</sup>	80 <sup>b</sup>	120 <sup>d</sup>	85 <sup>b</sup>	72 <sup>a</sup>	98 <sup>c</sup>	100 <sup>c</sup>	127 <sup>d</sup>	92 <sup>bc</sup>	5.1	NS	NS
Hemicellulose loss <sup>1</sup>	115 <sup>e</sup>	133 <sup>f</sup>	150 <sup>g</sup>	61 <sup>b</sup>	77 <sup>c</sup>	118 <sup>c</sup>	41 <sup>a</sup>	92 <sup>d</sup>	189 <sup>i</sup>	127 <sup>ef</sup>	172 <sup>h</sup>	200 <sup>i</sup>	14.3	**	**
Cellulose loss <sup>1</sup>	38 <sup>a</sup>	161 <sup>c</sup>	173 <sup>c</sup>	25 <sup>a</sup>	122 <sup>b</sup>	190 <sup>c</sup>	8 <sup>a</sup>	113 <sup>b</sup>	263 <sup>d</sup>	22 <sup>a</sup>	101 <sup>b</sup>	191 <sup>c</sup>	23.5	NS	***
Lignin loss <sup>1</sup>	170 <sup>b</sup>	253 <sup>d</sup>	289 <sup>e</sup>	130 <sup>a</sup>	249 <sup>d</sup>	305 <sup>e</sup>	131 <sup>a</sup>	179 <sup>b</sup>	221 <sup>c</sup>	157 <sup>b</sup>	182 <sup>b</sup>	230 <sup>c</sup>	16.9	NS	**
Residual ash <sup>1</sup>	76 <sup>a</sup>	86 <sup>cd</sup>	95 <sup>e</sup>	76 <sup>a</sup>	89 <sup>d</sup>	94 <sup>e</sup>	74 <sup>a</sup>	80 <sup>b</sup>	85 <sup>c</sup>	75 <sup>a</sup>	85 <sup>c</sup>	102 <sup>f</sup>	2.6	NS	**
Digestibility <sup>1</sup>	148 <sup>a</sup>	206 <sup>c</sup>	232 <sup>g</sup>	162 <sup>b</sup>	220 <sup>f</sup>	259 <sup>h</sup>	145 <sup>e</sup>	192 <sup>d</sup>	231 <sup>g</sup>	147 <sup>a</sup>	178 <sup>e</sup>	221 <sup>f</sup>	11.2	**	***

<sup>1</sup> g/kg dry matter basis, TOM - total organic matter; the values represent mean of six individual samples; different superscripts within column are significantly (P<0.05) different; CD - critical difference at 5% level; loss in TOM, 13.14; water soluble conc., 7.64; hemicellulose loss, 10.53; cellulose loss, 32.24; lignin loss, 25.01; residual ash, 3.72; digestibility, 7.14. SE - standard error; NS: non significant (P>0.05); A - level of significance for fungus type; B - level of significance for incubation time; \*\* P<0.05; \*\*\* P<0.001

days in cellulose degradation as compared to *P. fascicularia*, which degraded cellulose more in last 10 days.

All the fungi were able to degrade lignin; *P. brevispora* was the best lignin degrader and resulted in maximum lignin loss of 305 g/kg in 30 days of incubation. It was closely followed by *P. radiata* (289 g/kg), whereas *C. subvermispora* and *P. fascicularia* caused a lignin loss of 230 and 221 g/kg, respectively. *P. radiata* and *C. subvermispora* were relatively faster degraders of lignin and resulted in 170 and 157 g/kg loss in first 10 days of incubation, while *P. brevispora* was better in later stage of incubation, i.e. 20-30 days (Table 1).

In all the fungal treated substrates, pH declined sharply during first 10 days of incubation and fell to 3.5-5.4 as compared to uninoculated WS (pH 6.04). However, it increased slightly thereafter (Table 2). Residual ash content of

Table 2. Changes in pH profile of WS during its degradation by different fungi

Organisms	Days of incubation			
	0	10	20	30
<i>P. radiata</i>	6.04	3.56	4.85	4.94
<i>P. fascicularia</i>	6.04	5.42	5.53	5.79
<i>P. brevispora</i>	6.04	3.81	4.96	4.98
<i>C. subvermispora</i>	6.04	3.59	4.52	4.94

degraded WS increased with increase in incubation period and maximum resultant ash (in 30 days) was obtained from WS degraded by *C. subvermispora* (102 g/kg), which was followed by *P. radiata* (95 g/kg), *P. brevispora* (94 g/kg) and *P. fascicularia* (85 g/kg). The residual ash content resulting from the degradation of WS by different fungi was statistically significant ( $P < 0.05$ ) except *P. radiata* and *P. brevispora*.

WS treated with *P. brevispora* showed the highest *in vitro* digestibility of 259 g/kg followed by *P. radiata* (232 g/kg), *P. fascicularia* (231 g/kg) and *C. subvermispora* (221 g/kg) during 30 days, whereas the control WS showed 143 g/kg digestibility. Difference in *in vitro* digestibility resulting from the WS treated with different fungi was statistically significant ( $P < 0.05$ ) except *P. radiata* and *P. fascicularia*. After first 10 days, only *P. brevispora* was able to enhance the digestibility up to a significant level which continued during the entire incubation period while other fungi enhanced digestibility during 10-30 days of incubation.

Different fungi showed different laccase production profile. *P. brevispora* was the best laccase producer and resulted in a maximum laccase production during 20 days, which was followed by *P. fascicularia* during 30 days, *C. subvermispora* during 10 days while laccase production by *P. radiata* was minimum as compared to other fungi (Figure 1).

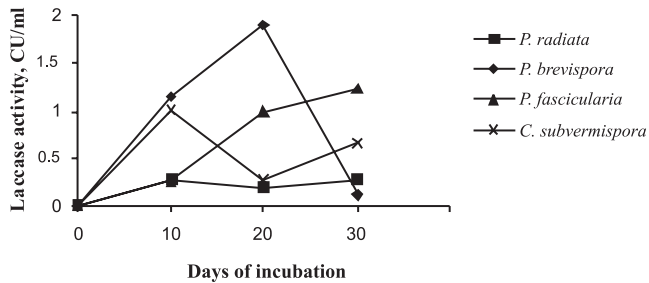


Figure 1. Laccase production by different white rot fungi during solid state fermentation of wheat straw for 30 days

*C. subvermispora* was the richest in chitin content (320 g/kg), which was followed by *P. fascicularia* (305 g/kg), *P. radiata* (247 g/kg) and *P. brevispora* (220 g/kg).

## DISCUSSION

Chemistry of lignin confers resistance to wood and other agricultural residues against microbial attack. However, white rot fungi have got the necessary potential to degrade lignin because of their well defined ligninolytic enzyme system, which make them suitable candidates for delignification of forage crops to improve their digestibility (Zadrazil, 1977). Invariably these fungi also degrade cellulose and hemicellulose along with lignin. The loss of these components from the feed is not economically desirable because less biomass is available for animal feed. In an earlier study *Phanerocheate chrysosporium*, a common and widely studied fungus, degraded lignin but also caused a high loss in total organic matter (TOM) (Jung et al., 1992). To overcome this problem, selective ligninolysis needs to be carried out. In the present studies, all the white rot fungi promise to be a good choice of being selective lignin degraders with minimum loss in TOM.

From the experimental data, the lignin loss as caused by different fungi can be correlated with the enhancement of digestibility of substrate (Correlation coefficient = 0.915, which shows a very strong positive correlation). *In vitro* digestibility of wheat straw was maximum in *P. brevispora*, which caused maximum lignin loss and minimum loss in TOM. All the fungi were selective in lignin degradation and enhanced the *in vitro* digestibility accordingly. It also supported the earlier observations of Mukherjee and Nandi (2004) during their study on two *Pleurotus* species. After 20 days of incubation *P. brevispora* enhanced the *in vitro* digestibility up to 220 g/kg with 61 g/kg loss in TOM and there was no statistically significant difference when compared with enhancement in the *in vitro* digestibility caused by



*C. subvermispota* after 30 days of incubation with a simultaneous loss of 108 g/kg in TOM (Table 1). Thus, the use of *P. brevispora* is practically beneficial for improving the quality of wheat straw. Next to *P. brevispora*, *P. radiata* and *P. fascicularia* gave almost similar results. *P. radiata* degraded more lignin than *P. fascicularia* but there was no statistically significant difference in the *in vitro* digestibility of substrate treated by these fungi. Both the *Phlebia* species had same impact on the digestibility improvement, but economically *P. fascicularia* may prove to be more suitable candidate because of minimum loss in TOM and faster rate of digestibility enhancement. Martínez et al. (2005) reported that lignin and hemicellulose were attacked initially while the cellulose degradation rate increased later, which is in consonance with the present study. Initially all the fungi consumed simple components which did not give any significant difference in digestibility except *P. brevispora*. Concentration of water soluble components depends upon the degradation of complex compounds like cellulose and hemicellulose, which are converted into lower molecular weight components. However, water solubles are not directly related to digestibility though may be significant nutritionally. *P. radiata* released maximum water solubles but it was unable to give a proportional enhancement of *in vitro* digestibility in consonance with earlier findings of Rolz et al. (1986) with lemon grass and citronella bagasse as substrate and 12 white rot fungi.

In line with maximum ligninolysis, *P. brevispora* gave the highest laccase yield followed by *P. fascicularia* and *C. subvermispota*. Though, laccase activity was minimum in *P. radiata* but it degraded the lignin in a very selective and efficient manner, which shows that the lignin degradation also depends upon other ligninolytic enzymes, i.e. manganese peroxidase and lignin peroxidase, etc. (Arora et al., 2002).

Chitin is a basic structural component of fungal cell wall. Presence of fungal mycelia in these plant cells thus increase the chitin content, which may not be easily digested by ruminants. Amount of chitin depends upon the fungal species and its growing conditions. Thus, the chitin content may affect the digestibility of feed. Chitin may not be of much nutritional value to ruminants but its content can affect the digestibility of the fungal treated substrate. Maximum chitin was estimated in *C. subvermispota*, which showed the lowest increase in digestibility, while the fungus *P. brevispora* having the lowest chitin content enhanced the *in vitro* digestibility to its maximum extent, which is in the consonance with the study of Asiegbe et al. (1994). Other fungi also showed the relation between their chitin content and digestibility. *P. floridensis* also enhances the digestibility of WS up to 250 g/kg with a minimum loss of TOM, this fungus also has a lower amount of chitin (data not shown). *C. subvermispota* and *P. radiata* also degraded lignin selectively making them suitable choice with specific reference to pulping industry (Yaghoubi et al., 2008) while *P. brevispora* may turn out to be better fungus for practical use to improve the digestibility of wheat straw.

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