



Optimization of Solid State Fermentation of Mustard (*Brassica campestris*) Straw for Production of Animal Feed by White Rot Fungi (*Ganoderma lucidum*)

A. K. Misra*, A. S. Mishra, M. K. Tripathi, R. Prasad, S. Vaithyanathan and R. C. Jakhmola
Division of Animal Nutrition, Central Sheep and Wool Research Institute, Avikanagar-304501, India

ABSTRACT : The objective of the experiment was to determine the optimum cultural [moisture levels (55, 60 and 70%), days of fermentation (7, 14 and 21), temperature (25 and 35°C) of incubation] and nutritional parameters (urea addition (0 and 2%) and variable levels of single super phosphate (0.25 and 0.50% SSP)) for bio-processing of the mustard (*Brassica campestris*) straw (MS) under solid-state fermentation (SSF) system. The performance of SSF was assessed in terms of favorable changes in cell wall constituents, protein content and *in vitro* DM digestibility of the MS. Sorghum based inoculum (seed culture) of *Ganoderma lucidum* to treat the MS was prepared. The 50 g DM of MS taken in autoclavable polypropylene bags was mixed with a pre-calculated amount of water and the particular nutrient in the straw to attained the desired levels of water and nutrient concentration in the substrate. A significant progressive increase in biodegradation of DM ($p < 0.001$), NDF ($p < 0.01$) and ADF ($p < 0.05$) was observed with increasing levels of moisture. Among the cell wall constituents the loss of ADF fraction was greatest compared to that of NDF. The loss of DM increased progressively as the fermentation proceeded and maximum DM losses occurred at 28 days after incubation. The protein content of the treated MS samples increased linearly up to the day 21th of the incubation and thereafter declined at day 28th, whereas the improvement in *in vitro* DM digestibility were apparent only up to the day 14th of the incubation under SSF and there after it declined. The acid detergent lignin (ADL) degradation was slower during the first 7 days of SSF and thereafter increased progressively and maximum ADL losses were observed at the day 28th of the SSF. The biodegradation of DM and ADL was not affected by the variation in incubation temperature. Addition of urea was found to have inhibitory effect on fungal growth. The effect of both the levels (0.25 and 0.50) of SSP addition in the substrate, on DM, NDF, ADF, cellulose and ADL biodegradation was similar. Similarly, the protein content and the *in vitro* DM digestibility remain unaffected affected due to variable levels of the SSP inclusion in the substrate. From the results it may be concluded that the incubation of MS with 60 percent moisture for 21 days at 35°C with 0.25 percent SSP was most suitable for MS treatment with *Ganoderma lucidum*. Maximum delignification, enrichment in the protein content and improvement in *in vitro* DM digestibility were achieved by adopting this protocol of bioprocessing of MS. (**Key Words :** Straw, Mustard, Bioprocessing, Delignification, Digestibility, Fungi, Fermentation, Treatment, *Ganoderma lucidum*)

INTRODUCTION

The mustard straw (MS) is a main crop residue of the semi arid region of India and around 14.0 million tones of MS are being produced annually (Misra et al., 2000). The MS contains very low amount of protein and large proportion of cell wall constituents (Mishra et al., 1996), which renders it unpalatable and poorly digestible to the ruminants (Mishra et al., 2004). The cellulose content in MS is about 45 percent (Vaithyanathan et al., 2003), but this vast amount of energy source is locked in ligno-

cellulosic complex and remains un-accessible to ruminal microbes for degradation (Chaudhry, 1998). In our laboratory, attempts to improve the nutritive value of MS have been made through various chemicals (Misra et al., 1995; Misra et al., 2000; Vaithyanathan et al., 2003) and variable degree of success has been achieved. Apart from the chemicals, the biological method to improve the quality of straw and other low-grade roughages is drawing much attention due to its specificity and simplicity in improving the nutritive value without having many disadvantages of chemical pretreatment (Fahey et al., 1993). A mixed response in improvement of *in vitro* dry matter (DM) digestibility (IVDMD) (10-20 percentage units) or negative responses depending on the type of crop residue, botanical fractions, fungal species and the preparation of substrate prior to fungal decay, were obtained with white rot fungi

* Corresponding Author: A. K. Misra, Plant and Animal Relationship Division, Indian Grassland and Fodder Research Institute, Jhansi-284003, India. Tel: +91-517-2730128, Fax: +91-517-2730833, E-mail: asimumisra@rediffmail.com

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Table 1. List of cultural and nutritional variables for optimization of solid-state fermentation process for mustard straw

Parameters	Variables
Cultural conditions	
Moisture (%)	55, 60, 70
Temperature (°C)	25, 35
Period of incubation (days)	7, 14, 21, 28
Nutritional conditions	
Nitrogen (through fertilizer grade urea (%))	0, 2
Single super phosphate (fertilizer grade (%)) containing: Phosphate and sulphate (16%)	0.25, 0.50

(WRF) (Karunanandaa and Varga, 1996; Agosin et al., 1987; Reid, 1989). The normal method of cellulosic material degradation by WRF is for the cellulose and lignin to be attacked simultaneously (Ander and Eriksson, 1978), resulting DM loss during the process of fermentation and rendering the straw fibre more accessible to rumen enzymes for subsequent digestion, thus providing the scope for utilization of ample quantity of DM as animal feed which would otherwise be decayed in the field. The extent of improvement in straw quality fermented with WRF is regulated by the relative degradation of lignin and carbohydrates (Holocellulose) besides the protein production (Kamra and Zadrazil, 1988), which in turn regulated by various fermentation parameters (nutritional and cultural) and varies with the type of the substrate (Tripathi and Yadav, 1992). Hence, in the present experiment, the bioprocessing conditions for the solid state fermentation (SSF) by *Ganoderma lucidum* (GL) strain of WRF, were optimized by varying the fermentation parameters. The performance of SSF was assessed in terms of favorable changes in dry matter (DM), cell wall constituents, crude protein (CP) content and *in vitro* DM digestibility of the MS.

MATERIALS AND METHODS

The study was carried out at Central Sheep and Wool Research Institute, Avikanagar, located at 26° 17'N latitude, 75° 28'E longitude and 320 m above sea level. All the incubations were carried out under temperature-controlled conditions.

Bio processing of straw

Pure culture of *Ganoderma lucidum*, a white rot fungus obtained from the Forest Research Institute, Dehradun, India, was maintained on sabouraud dextrose agar containing mycological peptone (10 g/L), dextrose (40 g/L) and agar (15.0 g/L) and sub cultured every month. Sorghum based inoculum (seed culture) of GL to treat the MS was prepared by growing the fungus (25°C for 7 days) on sterilized sorghum grains (supplemented with 2% gypsum (calcium sulphate) and 4% lime (calcium carbonate) in glass conical flask (250 ml capacity) closed with cotton

plugs. The MS used for study was obtained from a nearby farm and contained upper half portion of the plant. Mustard straw was chopped (2-3 cm) and sieved to remove dust and smaller particles. The 50 g DM of mustard straw taken in an autoclavable polypropylene bag (12"×8"), was mixed with a pre-calculated amounts of water, nitrogen, phosphorus and sulfur to attained the desired levels of water and nutrient concentration in the substrate as per Table 1. The urea and single super phosphate were used to provide nitrogen, phosphorus and sulfur in the substrate. The bags containing straw DM for fermentation were plugged with cotton and then autoclaved. The contents of bags were cooled and inoculated aseptically with spawn (grain culture; 2% W/W) and incubated at 25°C and 35°C temperature for 7, 14, 21 and 28 days in a biological oxygen demand incubator (BOD) without turning during incubation.

Chemical evaluation of straw

The DM loss due to fermentation was calculated by drying (50°C till the constant weight is achieved) the contents of the bags before and after SSF process. The dried MS was then ground (1 mm mesh size) and analyzed for NDF, ADF, ADL (Van Soest et al., 1991) and CP content (N*6.25; AOAC, 1990). The loss or gain in the level of straw constituents, namely DM, cell wall (NDF, Neutral detergent fiber; ADF, Acid detergent fiber; cellulose and acid detergent lignin) constituents as a result of fermentation, was calculated as the difference between the amount (gram) constituent incubated and the amount (gram) constituent recovered after completion of solid-state fermentation. The changes in protein content due to fermentation were determined by Macro Kjeldahl method after extracting the fermented MS with water to remove non-protein nitrogen or undergraded urea. The extraction was carried out by mixing 5 g of fermented MS with 100 ml of distilled water followed by stirring, filtration through a sintered glass crucible and redrying. The *in vitro* DM digestibility (IVDMD) assay was carried out as per the procedure described by Tilley and Terry, (1963) and one Malpura ram (35 kg BW and 36 months old), fitted with rumen cannulae, was used in this experiment for rumen liquor. Ram was maintained on (*Cenchrus ciliaris*) straw-based diet (roughage to concentrate ratio, 65:35).

Table 2. Biodegradation of DM and cell wall constituents (g kg⁻¹ of original samples) and changes in protein content *in vitro* DM digestibility (%) of mustard straw incubated with white rot fungi (*Ganoderma lucidum*) under solid state fermentation at 55, 60 and 70% moisture levels

Moisture (%)	Dry matter	Neutral detergent fiber	Acid detergent fiber	Cellulose	Acid detergent lignin	Protein (%)	IVDMD (%)
55	46.30	65.76	138.74	68.94	39.65	3.75	29.96
60	56.14	76.06	139.50	63.63	48.01	4.04	30.62
70	61.79	78.78	145.99	63.22	41.12	4.06	30.18
P	***	**	*	**	*	**	NS
SEM	0.44	1.70	2.21	2.64	2.72	0.03	0.20

The original mustard straw had values of: NDF, 722 g kg⁻¹; ADF, 628 g kg⁻¹; Hemi cellulose, 94 g kg⁻¹;

Cellulose, 495 g kg⁻¹; lignin, 108 g kg⁻¹; crude protein, 29.9 g kg⁻¹; *in vitro* DM digestibility (%), 22.80.

P = Statistical significance; SEM = Standard error of means.

* p<0.05; ** p<0.01; *** p<0.001. NS = Non-significant.

Table 3. Fermentation losses of dry matter and cell wall constituents (g kg⁻¹ of original samples) and changes in protein content and *in vitro* DM digestibility of mustard straw at 7, 21 and 28 days incubation under solid state fermentation with white rot fungi (*Ganoderma lucidum*)

Days of incubation	Dry matter	Neutral detergent fiber	Acid detergent fiber	Cellulose	Acid detergent lignin	Protein (%)	IVDMD (%)
7	36.10	44.34	107.26	40.73	34.39	3.95	24.12
14	48.01	53.37	109.10	54.90	60.86	4.52	32.34
21	60.68	75.00	157.57	74.64	71.16	3.72	38.21
28	74.13	121.40	193.03	117.45	84.16	3.62	26.34
P	***	***	***	***	***	***	***
SEM	0.50	1.96	2.55	3.05	7.76	0.03	0.24

P = Statistical significance; SEM = Standard error of means; *** p<0.001.

Statistical analysis

Data were analyzed in complete randomized block design with 2×2×3×4 factorial arrangements of treatments in triplicates using SPSS Base 10 (SPSS software products, USA) package. The group differences were compared by using Duncan's Multiple Range Test (Duncan, 1955). The factors were the moisture levels, incubation temperature, period of incubation, urea and single super phosphate levels. The experiment generated 288 samples for evaluation of changes in DM, cell wall, protein and *in vitro* DM digestibility.

RESULTS AND DISCUSSION

The results of effects of moisture levels on biodegradation of DM and cell wall constituents are presented in Table 2. A significant progressive increase in biodegradation of DM (p<0.001), NDF (p<0.01) and ADF (p<0.05) was observed with increasing levels of moisture. The effect of 60 and 70% moisture addition on biodegradation of NDF content was similar. The degradation of acid detergent fiber was higher at 70% moisture content of the substrate. In case of cellulose, the maximum losses occurred at 55% moisture level. Among the cell wall constituents the loss of ADF fraction was greatest compared to that of NDF. The maximum (p<0.05) bio-delignification was observed at 60 percent moisture content. Results indicated that the moisture content in the

substrate is a critical variable and must be controlled at an optimum level (Kamra and Zadrazil, 1988). Excessive moisture hinders the gas exchange and creates anaerobic conditions, whereas insufficient water content in the substrate limits the fungal activity. Further, the optimum ratio of solid to liquid in solid-state fermentation system depends upon the quality, particles size and water holding capacity of the substrate (Zadrazil and Brunnert, 1981). MS treated with 60 and 70 percent moisture showed improvement in (p<0.01) in CP over the substrate having 55 percent as well as untreated MS (2.99% CP). The loss of DM increased progressively as the fermentation proceeded and maximum DM losses occurred at 28 days after incubation. Similar losses have been reported for wheat and rice straw treated with *C. fimevarius* (Rai et al., 1989; Singh et al., 1990). The loss of DM was obviously due to lack of preferential lignin degradation during substrate decomposition (Zadrazil et al., 1982). An increase in protein content and *in vitro* DM digestibility was observed on fungal treatment of MS with GL (Table 3) over the untreated samples. The protein content of the treated samples increased linearly up to the day 21st of the incubation and thereafter declined at day 28th (Table 3). Similarly, the improvement in *in vitro* DM digestibility were apparent only up to the day 21st of the incubation under SSF and there after it reduced. However, the exact reason for this unexpected reduction in *in vitro* DM digestibility is not known. The progressive increase in DM losses coupled with

Table 4. Effect of incubation temperature on loss of dry matter, cell wall constituents (g kg⁻¹ of original samples), protein content and *in vitro* DM digestibility of mustard straw incubated with white rot fungi (*Ganoderma lucidum*) under solid-state fermentation

Temperature (°C)	Dry matter	Neutral detergent fiber	Acid detergent fiber	Cellulose	Acid detergent lignin	Protein (%)	IVDMD (%)
25	54.97	88.87	163.91	87.43	111.06	3.84	24.42
35	54.49	58.99	117.57	56.43	105.16	4.07	36.09
P	NS	**	**	**	NS	**	**
SEM	0.36	1.39	1.80	2.16	0.30	0.02	0.17

P = Statistical significance; SEM = Standard error of means; ** p<0.01; NS = Non-significant.

Table 5. Effect of addition of nitrogen through urea in the substrate on loss of dry matter, cell wall constituents (g kg⁻¹ of original samples), protein content and *in vitro* DM digestibility of mustard straw incubated with white rot fungi (*Ganoderma lucidum*) under solid-state fermentation

Urea (%)	Dry matter	Neutral detergent fiber	Acid detergent fiber	Cellulose	Acid detergent lignin	Protein (%)	IVDMD (%)
0	67.67	114.26	175.42	100.19	42.43	4.79	28.26
2.0	41.79	32.79	106.07	43.67	23.42	3.12	32.25
P	***	***	***	***	*	**	**
SEM	0.36	1.39	1.80	2.16	5.48	0.02	0.17

P = Statistical significance; SEM = Standard error of means; ** p<0.05; NS = Non-significant.

lowered DM digestibility at later part of the fermentation (after 14 days) could be due to the utilization of available holocellulose thus leaving the lingo-carbohydrate complex, resistant to microbial attack (Zadrazil and Brunnert, 1981). Several workers have reported protein enrichment on fungal treatment (Singh et al., 1990; Gupta and Singh, 1991; Walli et al., 1991) of various straws. Dahia et al. (2004), observed an increase of 13.50 and 16.68 percent in protein content of wheat and gram straw, respectively treated with *C. finetarius* fungus. The production of fungal protein is highly associated with organic matter losses and various factors like pH, temperature, moisture, aeration, heat transfer, nature of fungi, types of substrate and duration of fermentation influence the efficacy of the SSF for protein enrichment (Zadrazil, 1987). Regarding the quality of N fixed up by the fungus, it has been argued that only part of the mycelial N may represent true protein with rest as chitin and nucleic acids (Zadrazil, 1987). ADL degradation, the main targeted constituent of cell wall through bio processing, was slower during the first 7 days of SSF and thereafter increased progressively and maximum ADL losses were observed at the day 28th of the SSF. This may be attributed to more utilization of cell contents, cellulose and hemicellulose by the fungus to meet the energy requirement during early phase of fermentation, whereas more degradation of lignin thereafter, indicated that fungal enzyme might have been released in the biomass, which attacked lignin along with other polysaccharides. The biodegradation of DM and ADL was not affected by the variation in incubation temperature (Table 4). However, in case of NDF, ADF and cellulose, the incubation temperature caused significant variations in biodegradation of these fractions and comparatively lesser losses were observed at 35°C compared to 25°C temperature of incubation.

Similarly the maximum increase in protein content and *in vitro* DM digestibility values were observed in the samples incubated at 35°C temperature. Exceeding beyond 34°C to 42°C range of temperature leads to either contamination with other types of fungi or slow down the fermentation process, if the temperature is below 34°C (Rai et al., 1988). Initial temperature below 32°C in SSF increases the lag time.

Results on effect of addition of nitrogen through urea on biodegradation of DM and different fiber constituents of mustard straw under solid-state fermentation are presented in Table 5. Addition of urea adversely affected the protein enrichment of the MS due to inhibitory effect on growth of fungal biomass, as the treatment combinations having urea did show very poor mycelia development and resulted in poor biodegradation characteristics and hence the delignification. Surprisingly the higher *in vitro* DM digestibility coefficients were associated with the fermented MS samples having urea. The reason might be the readily available nitrogen source (from the undegraded urea) to the microbes of the *in vitro* system resulting higher microbial activity and enhanced digestibility. In case of SSP addition in the substrate, the effect of both the levels (0.25 and 0.50) on DM, NDF, ADF, cellulose and ADL biodegradation was similar. Similarly the protein content and the *in vitro* DM digestibility remain unaffected due to variable levels of the SSP, indicating that 0.25 percent SSP inclusion in the substrate could met the requirement of fungus for sulfur and phosphorus.

CONCLUSIONS

Crop residues fermented with various fungi under solid-state fermentation may be converted into protein-enriched

Table 6. Effect of addition of sulphur and phosphorus through single super phosphate in the substrate on loss of dry matter, cell wall constituents (g kg⁻¹ of original samples), protein content and *in vitro* DM digestibility of mustard straw incubated with white rot fungi (*Ganoderma lucidum*) under solid-state fermentation

Single super phosphate addition (%)	Dry matter	Neutral detergent fiber	Acid detergent fiber	Cellulose	Acid detergent lignin	Protein (%)	IVDMD (%)
0.25	55.13	72.16	137.58	69.89	35.92	3.96	30.31
0.50	54.35	74.90	143.91	73.97	35.30	3.94	30.20
P	NS	NS	NS	NS	NS	NS	NS
SEM	0.35	1.40	1.80	2.16	1.48	0.17	0.02

P = Statistical significance; SEM = Standard error of means; ** p<0.01; NS = Non-significant.

feeds. However, the production of fungal protein is highly associated with organic matter losses. Various factors like pH, temperature, moisture aeration, heat transfer, nature of fungi, types of substrate and duration of fermentation are critical and influence the efficacy of the SSF. The performance of the solid-state fermentation system of MS with variable cultural (moisture level, days of fermentation, temperature of incubation) and nutritional parameters (urea addition and variable levels of single super phosphate) indicated that the incubation of MS with 60 percent moisture for 21 days of at 35°C with 0.25 percent SSP is most suitable for MS treatment with *Ganoderma lucidum*. Addition of urea in the substrate was found have inhibitory effect on the growth of the fungus (*Ganoderma lucidum*). Maximum delignification, enrichment in the protein content and improvement in *in vitro* DM digestibility were achieved by adopting this protocol of bioprocessing of MS.

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