6. Summary

Different lignocellulosics are generally used as raw material in several industries e.g. pulp and paper, renewable energy etc. Agricultural residues, mainly cereal straws generally used as animals feed are of poor nutritive quality as compared to green fodder. Major components of these residues comprises of water soluble part, pectin, hemicellulose, cellulose and lignin. Different polysaccharides e.g. cellulose and hemicellulose are protected by lignin which is a complex heterogeneous phenolic polymer, and provides strength to plant. Lignin is also resistant to most of the microbial and animal enzymatic actions.

Among a variety of microorganisms, white rot fungi are good lignin decomposers and some of these are well known for selective ligninolysis, thus enhancing the availability of holocellulose and making it more easily digestible by ruminants. Beside this, the growth of proteinaceous fungal mycelium contributes to the nutritive value of feed.

Several microorganisms are used to ferment agro residues under solid state conditions for the production of different industrially important enzymes as well as for the bioconversion of residues into nutritive animal feed. Production of enzymes by filamentous fungi during solid state fermentation has fascinated many workers. Solid state fermentation constitutes an interesting alternative to submerged fermentation, since metabolites so obtained are more concentrated and purification procedures are less costly. It involves low moisture content, which also minimizes the chances of bacterial contamination. A lot of work has been done on the production of different lignocellulolytic enzymes by white rot fungi and its optimization studies; but only a scant literature is available on the optimization of biodelignification and enhancement in digestibility of the resulting agro residues. Thus, the study was planned with following objectives:

- To study the selective ligninolytic ability of some white rot fungi
- To study the correlation of ligninolytic ability of white rot fungi and their role in improvement of *in vitro* digestibility
- To enhance the digestibility of agro residues (wheat straw and rice straw)
Enhancement in nutritive value of feed in terms of *in vitro* digestibility (IVD) and total protein content.

Seven white rot fungi including *Ceriporiopsis subvermispora* (FP-90031), *Daedalea flavida* (MTCC-145), *Phanerochaete chrysosporium* (BKM-F-1767), *Phlebia brevispora* (HHB-7030), *Phlebia fascicularia* (FP-70880), *Phlebia floridensis* (HHB-5325), *Phlebia radiata* (MJL-1198), were selected for the study.

Lignocellulosic residues: wheat straw (WS) and paddy straw (PS) were obtained from the fields of Guru Nanak Dev University, Amritsar, Punjab, India. To study the effect of different geographic locations and climatological conditions, samples of WS and PS were collected from three different Indian locations i.e. north western zone (NWZ), north eastern zone (NEZ) and central zone (CZ). The experiments were performed on the following lines:

- Reference strains of white rot fungi selected on the basis of their better ligninolytic ability were screened for their selective ligninolysis of wheat straw and paddy straw under solid state conditions.
- Correlation between total organic matter loss, holocellulose degradation, selective ligninolysis, lignocellulolytic enzyme production, enhancement in IVD and nutritive quality of wheat and paddy straw was established.
- Time profile of lignocellulosic residues degradation was studied i.e. 30 days for WS and 60 days for PS.
- The effect of different geographic locations and climatological conditions on lignocellulosic degradation was studied by using wheat and paddy straws collected from northern, eastern and central zones of India.
- Effect of the supplementation of inorganic and organic nitrogen rich sources on ligninolysis, IVD and nutritive quality of straws were studied and the conditions were optimized using response surface methodology.
- Crude protein, amino acids and total phenolic contents (TPC) were estimated in the degraded straw and compared with undegraded control.
- The experimental set up was scaled up from 5g to 200g batches of straw to see the efficacy and efficiency of the best selected organisms i.e. *P. brevispora* and *P. floridensis*. 
Safety evaluations of the resultant biodegraded material were carried out using Ames test for mutagenecity and MTT assay for cytotoxicity.

**Biodegradation of wheat straw (WS)**

Solid state fermentation of wheat straw was carried out using seven white rot fungi and these were examined for their potential to degrade different plant cell wall components. After fungal treatment of WS, loss in total organic matter (TOM), hemicellulose, cellulose and lignin with associated water soluble part and ash content were analyzed. Lignocellulolytic enzyme production (laccase, xylanase and CMCase) were also estimated. Changes in pH and *in vitro* digestibility were recorded at 10, 20 and 30 days of incubation along with all other parameters mentioned above.

**Lignocellulose degradation**

*P. chrysosporium* grew vigorously on WS and caused maximum loss in TOM (54 %), liberated 11.7 % of water solubles and also degraded sufficient amount of fibers i.e. hemicellulose (59.2 %), cellulose (67.2 %) along with lignin (47.6 %), during 30 days of solid state fermentation. *P. chrysosporium* increased the IVD of WS from 17.2 to 21 %. *C. subvermispora* degraded 16.4 % of TOM and released 18.2 % of water solubles. Maximum cellulose loss was 28.5 % while a similar amount of lignin and hemicellulose (25.2 %) were degraded by the fungus with an increase in IVD from 17.2 to 25.4 %. Ash content also increased during the fermentation and was maximum on 30th day (10 %). *D. flavida* caused a maximum loss of 22 % in TOM with a lignin loss of 18.7 %. Cellulose and hemicellulose loss was 40.7 % and 21 % respectively. Water solubles increased to a maximum concentration of 8.5 % on 20th day while ash content increased up to 8.6 % during 30 days of incubation. However, *D. flavida*, was unable to enhance the *in vitro* digestibility.

Of the different *Phlebia* spp. tested, *P. brevispora* degraded 16.3 % of TOM and liberated maximum water solubles on 30th day (22 %). A maximum of 29 % hemicellulose, 17 % of cellulose and 30.6 % of lignin was degraded by the fungus during 30 days of incubation period. A respective enhancement recorded in ash content and IVD was up to 9.4 % and 28.7 %. *P. fascicularia* degraded almost similar amount of TOM as *P. brevispora* (16.7 %). Maximum water solubles (15 %) were liberated on 30th day with a loss of 29.4 % of hemicellulose and 22.2 % of cellulose. A maximum lignin of 23 % was degraded accompanied by an increase in ash content and IVD to 9.5 and 28.4 %, respectively. *P. floridensis* also caused a loss of 16.3 % in TOM. It
liberated 16.7 % of water solubles and degraded 17 % of hemicellulose, 22.6 % of cellulose and 27.5 % of lignin. Ash and IVD was enhanced up to 9.5 and 27 % respectively. *P. radiata* was comparatively slow in its growth during degradation of WS and caused a loss of 9.8 % in TOM. Cellulose and hemicellulose were degraded up to 26.3 and 19.2 %, respectively, with a loss of 28 % in lignin during 30 days. The maximum water solubles liberated were 15.2 %. Ash content also increased slightly. *P. radiata* raised the IVD of WS from 17.2 to 24.5 %.

A decline in pH of WS degraded by different fungi was recorded. Initially pH declined and rose gradually up to 20 days and then remained almost constant during 20 to 30 days, which was lower than the pH of undegraded WS.

*Lignocellulolytic enzymes*

Lignocellulolytic enzymes *viz.* laccase, CMCase and xylanase were studied during straw degradation. Except *P. chrysosporium*, all the organisms were able to produce laccase and maximum production was recorded on 10th day in *P. floridensis*, which was followed by *C. subvermispora, P. brevispora, P. fascicularia* and *P. radiata* while laccase production by *D. flavida* was minimum as compared to other fungi listed above. All the fungi were able to produce xylanase and CMCase. *P. chrysosporium, C. subvermispora* and *P. fascicularia* produced reasonably better xylanase and CMCase as compared to other fungi.

*Nutritional value*

In comparison to control (17.2 %), maximum enhancement in IVD was obtained in the WS degraded by *P. brevispora* (28.7 %) and *P. fascicularia* (28.4 %), followed by *P. floridensis* 27 %, *C. subvermispora* (25.4 %) and *P. radiata* (24.5 %) while in *P. chrysosporium* it was minimum (21 %). However, no enhancement in IVD could be recorded in WS degraded by *D. flavida*. Crude protein, amino acid content, total phenolic contents and antioxidant activity were also significantly higher in all the degraded straw and were maximum in *P. chrysosporium* followed by *P. floridensis*. In WS degraded by different fungi, the antioxidant activity was almost double, while about 1.5 folds enhancement in crude protein, amino acids and TPC was recorded.

**Biodegradation of paddy straw (PS)**

Solid state fermentation of paddy straw was carried out with seven white rot fungi. The degradation process was extended upto 60 days because of the slower growth of fungi on the substrate. All the tested fungi were able to grow on PS under the
experimental conditions. All the parameters used to assay WS quality were also used for PS analysis.

**Lignocellulose degradation**

Of the different fungi, *P. chrysosporium* caused maximum loss in total organic matter (TOM) of 46.4% and liberated maximum water solubles on 20th day (19.6%), during 60 days of incubation. The organism was fast growing and degraded all the components i.e. hemicellulose (51%), cellulose (52%) and lignin (39.4%). *C. subvermispora* degraded 16.8% of TOM, 28.7% of hemicellulose, 30.8% cellulose and 18.8% lignin during 60 days of incubation while maximum water solubles (14.8%) were liberated on 30th day. *D. flavida* followed *C. subvermispora* and degraded 15.6% of TOM and liberated 13% of water solubles in 60 days. *D. flavida* degraded a maximum of 12.5% hemicellulose, 14.2% cellulose and 19.4% of lignin, during the same period.

Among different *Phlebia* spp., *P. radiata* caused a loss of 13.9% in TOM, 21.6% in hemicellulose, 13.4% in cellulose, 22.8% in lignin during 60 days of incubation while liberated almost similar amount of water solubles (12.5%) on 30th and 60th day. *P. floridensis* and *P. fascicularia* caused almost similar loss (10.8%) in TOM. *P. fascicularia* liberated a maximum of 12.5% of water solubles on 20th day and degraded 19.6% of hemicellulose, 10.3% of cellulose and 21% of lignin during 60 days of incubation. *P. floridensis* liberated 12% water solubles and degraded 18.8% of hemicellulose, 11.4% of cellulose and 21.8% of lignin. *P. brevispora* caused the lowest loss (9.8%) in TOM. Maximum water solubles (11.6%) were liberated during 60 days of incubation and degraded 12.8% hemicellulose, 8.7% of cellulose and 20% of lignin.

**Lignocellulolytic enzymes**

All the organisms were able to produce different enzymes (laccase, CMCase and xylanase) during degradation of PS; except *P. chrysosporium*, which was not able to produce detectable laccase during the experimental conditions. Maximum laccase was produced by *P. floridensis* followed by *P. radiata*, *C. subvermispora* and *P. brevispora* on 30th day of incubation, respectively. *D. flavida* and *P. fascicularia* were lower in laccase production. *P. radiata* produced the maximum xylanase on 30th day, while *P. chrysosporium* produced a maximum amount of xylanase on 20th day, which decreased
gradually during further incubation and the profile was similarly followed by *C. subvermispora*. *P. fascicularia* produced maximum xylanase on 30th day, whereas *P. brevispora* and *P. floridensis* produced maximum xylanase on 60th day. CMCase was maximally produced by *P. chrysosporium* on 30th day and similarly followed by *C. subvermispora*, *P. radiata* and *P. floridensis*, while *P. brevispora* and *D. flavida* had almost similar enzyme production on 30th and 60th day. In the case of *P. fascicularia*, CMCase production gradually increased up to the end of experiment.

**Nutritional value**

*P. chrysosporium* and *P. brevispora* maximally enhanced the IVD of degraded PS from 18.5 to 25 %, while the *in vitro* digestibility ranged from 23 – 25 % for remaining fungi, during 60 days of incubation period. Nitrogen content, crude protein and amino acids content increased at least 1.4 folds in PS degraded by different fungi. Total phenolic contents and antioxidant activity also increased significantly ranging from 2 to 5 folds in degraded PS. Except TPC and antioxidant activity, *P. chrysosporium* enhanced maximum nutritional value but a huge biomass loss (TOM) was the limiting factor for the use of *P. chrysosporium*.

**Degradation of lignocellulosic residues obtained from different geographic locations**

Wheat and paddy straw obtained from three geographically different regions showed certain variations in their biochemical constituents. Water solubles were higher in sound WS and PS collected from NWZ, PS of this region had maximum cellulose content. WS of NEZ had maximum cellulose content while WS of CZ and PS of NEZ had higher hemicellulose content. Lignin contained was maximum in WS obtained from CZ (24 %) followed by NEZ (21 %) and NWZ (20.5 %). IVD was maximum in WS obtained from NWZ (17.2 %) followed by NEZ (16.5 %) and CZ (14.5 %), respectively. PS obtained from NWZ and NEZ were lower in lignin (20.3-20.5 %) with higher IVD (18.5-18.8 %).

Five fungi (*C. subvermispora*, *P. brevispora*, *P. fascicularia*, *P. floridensis* and *P. radiata*) were selected to degrade WS and PS obtained from NEZ and CZ on the basis of their selective ligninolytic ability and potential for improving IVD. Different fungi showed different enzymatic and degradation profile and IVD, which might have been governed by differential chemical composition of lignocellulosic residues obtained from various regions. First part of the study was performed using WS and PS obtained
from NWZ, while for remaining two zones the experiments were designed and the important findings obtained are as follows:

**Wheat straw**

Wheat straw degradation obtained from NEZ: A maximum of 36 % lignin was degraded by *P. floridensis*, while the maximum IVD raised from 16.5 to 28.5 % by *P. radiata* and closely followed by *P. floridensis, C. subvermispora* and *P. brevispora* (27.5 - 27.8 %) during 30 days of incubation. Laccase and xylanase was maximally produced by *P. floridensis*, while CMCase production was maximally produced by *P. brevispora* on 10th day of incubation.

Wheat straw degradation obtained from CZ: Maximum lignin (30 %) was degraded by *P. floridensis* and *P. brevispora*. *P. brevispora* also increased maximum IVD from 14.5 to 26 %, closely followed by *P. floridensis* (25 %). Laccase activity was maximum in *P. brevispora*, while maximum xylanase and CMCase was produced by *C. subvermispora* and *P. radiata*, respectively on 20th day.

**Paddy straw**

Paddy straw degradation obtained from NEZ: A maximum of 19.5 % lignin was degraded by *P. floridensis* by simultaneous increase in IVD from 18.8 to 26.4 %, closely followed by *P. brevispora* (26 %). Laccase was produced maximally by *P. floridensis* on 20th day. *C. subvermispora* and *P. radiata* were found to be the best xylanase and CMCase producers, respectively.

Paddy straw degradation obtained from CZ: A maximum lignin (21 %) was degraded by *P. floridensis* and also raised maximum IVD of PS from 17.5 to 24.6 %. Maximum laccase and xylanase was also produced by *P. floridensis* on 20th and 30th days of incubation, respectively. *C. subvermispora* produced maximum CMCase on day 30th.

*P. brevispora* was more effective in degrading WS, while *P. floridensis* was the best for both the substrates, WS as well as PS with a promising approach in selective lignin degradation and enhancement of *in vitro* digestibility of straw, irrespective of the region. Both the fungi were thus selected for further experimentations.

**Effect of supplements on degradation of straw**

Eight different nitrogen rich supplements were selected to see their effect on the degradation of WS and PS by *P. brevispora* and *P. floridensis*. Among these, four inorganic i.e. ammonium chloride, ammonium nitrate, ammonium sulphate and
ammonium tartrate and four organic nitrogen rich supplements i.e. malt extract, soya bean meal, urea and peptone were supplemented to WS and PS to see their effect on biodelignification and IVD.

Among inorganic supplements, ammonium chloride accelerated ligninolysis and also enhanced IVD during solid state fermentation. On the other hand, organic nitrogen rich supplements were more specific and gave higher degree of ligninolysis and IVD depending upon the substrate and organism combination.

**Optimization studies**

Three independent variables (X₁: moisture content, X₂: ammonium chloride, X₃: malt extract or peptone or soya bean meal) were selected to enhance ligninolysis and IVD, on the basis of the results obtained from previous experiments. The optimization of the concentration of selected variables was done with the help of response surface methodology (RSM) using a Box–Behnken design. Each variable was studied at three different levels (1, 0 and -1). The experimental design included 17 flasks with five central points. Each 250 ml conical flask contained 5 g of wheat straw, 0 to 100 mg NH₄Cl/g, 0 to 100 mg malt extract/g and 1 or 2 to 10 ml of distilled water. The flasks were sterilized, inoculated, incubated and processed.

The mathematical relationship of response G (for each parameter) and independent variable X (X₁, moisture content; X₂, NH₄Cl; and X₃, malt extract) was calculated by the quadratic model equation.

\[ G = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \]  

Where, G is the predicted response; \( \beta_0 \), intercept; \( \beta_1 \), \( \beta_2 \), and \( \beta_3 \), linear coefficients; \( \beta_{11}, \beta_{22} \) and \( \beta_{33} \), squared coefficients and \( \beta_{12}, \beta_{13} \) and \( \beta_{23} \) interaction coefficients. MINITAB and statistical software package Design Expert® version 8.0 (Stat Ease, Inc, Minneapolis, USA) were used to obtain optimal working conditions and generate response surface graphs.

**Scaling up the experiment**

To scale up the experiment, 200g of straw was taken in an autoclavable plastic bag along with the optimized concentrations of supplements i.e. 5 ml distilled water, 20 mg NH₄Cl and 75 mg malt extract/g of WS for *P. brevispora* and 5.5 ml distilled water, 80 mg NH₄Cl and 25 mg SBM/g of PS for *P. floridensis*. The autoclaved bag was placed inside a surface sterilized plastic container (270 mm height, 240 mm upper
diameter and 180 mm lower diameter) and allowed to cool at room temperature in sterilized conditions. The bag was cut from the sides and inoculated with 25 mycelial discs (10 mm), grown on YGA plates for 7 days. The container was covered with sterilized thin plastic sheet having nine (8 mm) apertures plugged with cotton and after incubating it at 27 °C for 20 days, was processed with acetate buffer and the filtrate so obtained was used for enzyme analysis.

An increase in IVD of WS from 17.2 to 27.5 % (enhancement in IVD 60 %) and in PS from 18.5 to 25.8 % (enhancement in IVD 40 %) was observed in degraded biomass. Crude protein was almost double, while total amino acid content was about 4 times as compared to control. TPC was significantly higher in fermented samples (about 5 folds), which resulted in enhancement in antioxidant activity.

Chitin estimation revealed that the fungal cell wall contained 11 to 13 % (w/w) chitin and degraded straw (TOM) contained 0.7 to 0.8 % of chitin. Thus TOM was comprised of 5.8 to 6.5 % of fungal biomass.

**Safety evaluation**

In order to carry out the safety evaluation the extracts obtained from undegraded and degraded straw samples were also assayed for their mutagenic and cytotoxic effect. As the results obtained from Ames test, none of the extracts (undegraded and degraded) showed mutagenic activity. A few bacterial colonies (10-15) was observed on agar plates containing extracts, while more than 1000 colonies were observed on positive control (sodium azide) containing plate. Similarly, results obtained from MTT assay revealed that all the extracts were non cytotoxic and showed much higher absorbance (ranged from 0.550 to 0.675) as compared to positive control (0.107).

**Conclusions**

- All the white rot fungi efficiently degraded lignin.
- Except, *D. flavida*, all the tested fungi enhanced the *in vitro* digestibility of lignocellulosic residues, ligninolytic capability of the fungus can be used for other industrial processes.
- *Phlebia* spp. and *P. chrysosporium*, enhanced the *in vitro* digestibility almost to similar levels, while the loss in total organic matter was much lesser in all the fungi as compared to *P. chrysosporium*. 
• Biochemical constituents of straw varied with respect to their climatologically different geographical locations, which further governs the profile of fungal degradation of lignocellulose and enzyme production.

• *P. brevispora* and *P. floridensis* were the best organisms to provide a practically promising approach in selective lignin degradation and enhancement of *in vitro* digestibility of wheat straw and paddy straw.

• The present findings hold a promise for the production of industrially important enzymes by using an economic and easily available raw material (agricultural residues) under solid state conditions.

• The selected strains not only enhanced the digestibility of feed but also the protein content and antioxidant activities, thus, they hold the meaningful potential for their utilization for the production of high quality feeds.
**Future prospects**

As the bioprocessing of agricultural residues for animal feed is a vital system in the emerging field of feed biotechnology, new ideas for economic, natural and safe feed production are the demand of age. Cell free lignocellulolytic enzyme extract, their lyophilized and/or immobilized form can be used to treat straw for the enhancement in its nutritive quality. Use of genetic engineering to enhance selective ligninolysis may be of choice. The ligninolytic system can be studied under varied conditions to reduce the cost. Study of prokaryotic organisms along with fungi (synergistic relationship) may be studied to explore the field better. Nutritive quality of degraded straw can be further explored by analysing the samples for their essential amino acid content. Present findings may prove useful for designing an experimental setup to degrade lignin selectively and to improve the digestibility of other agricultural residues too. Studies on animals can be conducted to ensure the effectively of the feed in vivo and its practical utility in the field. The study thus provides an opportunity to exploit the situation further where more natural feeding conditions (setting up the bioreactors in the fields) can be simulated in the studies.