5. 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 makes them suitable candidates for delignification of agro residues. Ligninolytic potential of such fungi can be exploited in varied industrial fields such as pulp and paper, textile, food and feed industries. Presence of lignin in the plant cell wall affects the digestibility of lignocellulosic residues to be used as ruminant feed. Fungal delignification of such lignocellulosics not only enhances the digestibility of the feed but also improves their nutritional value (Okano et al., 2006). 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 then remains available for animal feed. *Phanerochaete chrysosporium*, a widely studied fungus, degrades lignin efficiently but also cause a high loss in TOM (Jung et al., 1992). To overcome this problem, selective ligninolysis is of great importance, be it in feed industry or pulp and paper industry. So, the present study is an effort to improve ligninolysis, enhance *in vitro* digestibility and to develop a biological process for enhancing the nutritive quality of lignocellulosic residues.

5.1. Correlation between lignin content and *in vitro* digestibility

Cell wall constituents of straw play an important role in determining its quality as animal feed. As evident from our observations, a strong negative correlation ($r = -0.966$) existed between lignin content and *in vitro* digestibility of undecayed PS samples, while a strong positive correlation ($r = 0.869$) was observed between lignin loss and *in vitro* digestibility of degraded PS. Scattered plot and Pearson’s correlation between lignin content and digestibility of wheat straw from different regions also showed a negative correlation ($r = -0.993$), which also support the concept that the lignin act as barrier during the digestion of lignocellulosics (Figure 5.1). On the other hand lignin degradation and digestibility showed a strong positive correlation ($r = 0.916$) during WS degradation. It strengthens the viewpoint that delignification plays an important role in improvement of the digestibility and feed value of straw. As also reported by Cohen *et*
al., (2002) that during selective lignin degradation, the cellulose is exposed and can be utilized by ruminants.

![Figure 5.1](image.png)

**Figure 5.1** Correlation between IVD and lignin content \((r = -0.993)\) of WS collected from different geographical locations

### 5.2. Degradation of lignocellulosic residues and IVD

As reported earlier (Martinz *et al.*, 2005), white rot fungi are known to attack initially on the hemicellulose lignin matrix, which was also clearly observed during the present study. The experiments used to study the profile of WS and PS degradation *vis a vis* their IVD (Table 4.2, 4.5); all the *Phlebia* spp. degraded higher amount of lignin selectively during the degradation of WS, though in PS hemicellulose and lignin both were degraded simultaneously during initial period, without much loss in cellulose. However, even during later period of incubation, cellulose loss remained low though hemicellulose and lignin degradation continued up to the end of the experiment to a reasonable extent. It was also reflected in their corresponding enzyme production profile. None of the *Phlebia* spp. were able to produce a detectable amount of lignocellulolytic enzymes during initial 10 days of incubation while *P. chrysosporium* and *C. subvermispora* were able to produce hemicellulase and cellulase during PS degradation (Figure 4.5, 4.6, 4.7). *P. chrysosporium* grew very fast on straw and degraded maximum hemicellulose followed by cellulose and then lignin in PS, while in WS it degraded almost equal amount of these fibres during first 10 days. The amount of cellulose degraded by *P. chrysosporium* was not achieved by any of the *Phlebia* spp.
even after the incubation of 60 days in case of PS and upto 30 days in case of WS (Table 4.2, 4.5).

During 60 days of paddy straw degradation, both the fungi *P. chrysosporium* and *P. brevispora* enhanced the maximum *in vitro* digestibility from 18.5 to 25 % with a respective loss of about 39.4 and 20 % in lignin (Table 4.5). This reflects that *P. chrysosporium* to be non selective in lignin degradation and thus degraded all the fibres simultaneously which resulted in more holocellulose loss and thus degraded a large amount (46.4 %) of TOM. On the other hand, *P. brevispora* degraded only 9.8 % TOM during the fermentation process, which accounts for more selective ligninolysis leaving behind a sufficient amount of TOM (Figure 5.2). Thus, a reasonable amount of easily digestible degraded biomass is available to the animal as feed. For practical purposes, higher TOM loss severely limits the use of *P. chrysosporium*, which is in consonance with earlier observations (Jung *et al.*, 1992). The degradation profile of *P. chrysosporium* indicates that the higher holocellulose degradation may be due to the higher production of xylanase and CMCase like enzymes. Similar remarks were also made during an earlier observation while working with *P. chrysosporium* under solid state conditions using cotton stalks and water hyacinth (Kerem *et al.*, 1992; Deshpande *et al.*, 2008).

Cellulase and hemicellulase like enzymes break down the complex polysaccharides into simple sugars, which contribute to major part of water solubles. The water soluble part provides easily digestible components which can be used by rumen microflora and contributes to the digestibility of straw. No direct correlation could be established between the water soluble content, fiber degradation and digestibility, as observed in earlier studies (Rolz *et al.*, 1986). It may also be possible that the fungi use the easily available sugars for their own growth also (Nigam *et al.*, 2009), which thus lead to underestimations of soluble components and thus may be interfering with the interpretations.

During first 10 days of paddy straw fermentation none of the studied fungus was able to enhance the *in vitro* digestibility up to a significant level, whereas three fungi (*C. subvermispora, P. fascicularia* and *P. floridensis*) showed somewhat lower digestibility than control (uninoculated) PS. However, all the fungi were able to enhance the digestibility during the later period of incubation. This observation showed that, initially
the fungi need some easily available components for their own growth thus limiting their availability for rumen microflora. The findings are in consonance with earlier observations of Zadrazil, (1977). As observed by Dorado et al., (1999), the composition of the water soluble fraction correlated with the extent of straw transformation. The initial fermentation stage (0–15 days) was characterized by the accumulation of water-soluble products from lignin degradation and fungal metabolism, the concentration of which tended to stabilize in the later stage (16–60 days). As observed during the present study, P. chrysosporium released maximum water solubles throughout the experiment however the in vitro digestibility increased gradually up to the end of the experiment. In most of the cases, the water soluble components were not liberated at a constant rate during fungal degradation, while the fiber degradation and digestibility was minimum on 10th day and increased steadily during further incubation (Table 4.2, 4.5). It suggests that digestibility also depends upon the availability of other polysaccharides as well as the structure of polymers (Bertrand et al., 2006) that are modified by the fungal enzymatic treatment.

Hemicellulose loss and in vitro digestibility was found to be positively correlated, which is in consonance with earlier studies on WS degradation by two different white rot fungi (Agosin et al., 1985). During an earlier study (Xu et al., 2010) on corn stover degradation by Irpex lacteus, lignin and hemicellulose were also selectively degraded over cellulose. As evident from the present study, the hemicellulose-lignin matrix was primarily attacked by white rot fungi, all the fungi degraded relatively higher amounts of lignin and hemicellulose during the degradation of WS obtained from NEZ. In the WS collected from the CZ, lignin and hemicellulose were degraded initially, but during the later period of incubation, cellulose degradation was more dominant than hemicellulose. In WS obtained from NWZ, P. brevispora degraded lignin and hemicellulose efficiently, while P. fascicularia degraded more hemicellulose than lignin. P. floridensis was also selective in lignin and hemicellulose degradation initially, but during the later period cellulose was more effectively degraded than hemicellulose (Table 4.2, 4.10, 4.11).

In the data obtained from RSM studies on WS degradation by P. floridensis, lignin and hemicellulose loss was also found to be highly correlated in a positive manner \( r = 0.942 \). This clearly indicated that in most of the varied carbon nitrogen
ratio the fungus attacked lignin hemicellulose matrix but the rate and efficiency of the
degradation was different. The Pearson’s correlation coefficient between TOM loss and
degradation of lignin ($r = 0.938$), was found to be maximum. A lignin loss of more than
23 % was observed in 9 out of 17 flasks, whereas cellulose and hemicellulose
degradation was limited to a maximum of 16 and 14 %, respectively. However, on the
other hand, RSM study on PS degradation by $P. floridensis$, demonstrated more than 15
% lignin loss in 11 out of 17 flasks, while cellulose or hemicellulose degradation was $\leq$
10 % (Table 4.20).

During an earlier experiment, out of the three constituents (hemicellulose,
cellulose, and lignin), hemicellulose and lignin showed the largest proportionate loss
after inoculation with the fungi $D. quercina$, $H. clathroides$, $P. laevigatus$, and $L$
obliquus. The other fungi showed the maximum loss in cellulose and hemicellulose
contents. It suggested that the digestion enhancement of wheat straw colonized by white
rot fungi is regulated by complex factors including the degradation of structural
carbohydrates and lignin (Jalč et al., 1998).

In the present study, high holocellulose loss was found to be closely related with
TOM loss; e.g. in the WS obtained from NEZ, $P. radiata$ degraded 24 % TOM, which
was the maximum loss caused by any fungus during the experiment on WS/PS
degradation collected from different geographic locations. This was the result of
simultaneous degradation, which degraded hemicellulose, 31 %; cellulose, 30 % and
lignin, 33 %. While looking at the ruminant digestibility of feed, it is important that the
strategy chosen should not cause much loss in TOM available to the animal.
Enhancement in the in vitro digestibility of WS by $C. subvermispora$, $P. radiata$, $P.$
brevispora, and $P. floridensis$ was statistically insignificant and ranged from 28.5 to
27.5 % whereas $P. brevispora$ caused approximately half loss in TOM (12 %) as
compared to $P. radiata$ (24 %), which reflects its higher selectivity towards ligninolysis.
On the other hand, $P. radiata$ showed selective ligninolytic behaviour during the
degradation of other straw samples (Table 4.10), which showed its specificity towards
substrate.

In all the experiments related to WS/PS degradation obtained from different
locations, TOM loss increased along with hemicellulose and cellulose degradation. $P.$
brevispora and $P. fascicularia$ degraded TOM upto its maximum in the straw collected
from NWZ, followed by NEZ and then CZ, while not much difference was observed in lignin degradation, but holocellulose followed the pattern of TOM loss (Table 4.2, 4.5, 4.10, 4.11, 4.12, 4.13). Similar observations regarding TOM loss have been reported earlier during the degradation study of oat straw and alfalfa stems with *Phanerochaete chrysosporium* (Jung et al., 1992).

Correlation between TOM loss and lignin loss was higher ($r = 0.955$) as compared to both the polysaccharides ($r = 0.921$ and 0.908 for cellulose and hemicellulose, respectively), which showed that the lignin loss contributed more in TOM loss in comparison to hemicellulose and cellulose; thus, giving a selective ligninolysis of the PS. Correlation between ligninolysis, loss in TOM and IVD of PS is illustrated in figure 5.2. The lignin loss was relatively lesser as compared to the loss in TOM during PS degradation by *P. chrysosporium*, while in all other cases it was relatively higher than TOM loss and clearly reflected selectivity towards lignin degradation by all *Phlebia* spp.

![Graph showing correlation between TOM loss, lignin loss, and IVD for different organisms](image)

**Figure 5.2** Loss in TOM, loss in lignin and IVD of PS degraded by different white rot fungi during 60 days of incubation
All the Phlebia spp. were selective in lignin degradation of the PS obtained from NWZ and degraded about 20 to 23 % of lignin, while none of the fungi could degrade more than 20 % of lignin in PS collected from NEZ (Table 4.5, 4.12). This may be due to variation in the constituents and concentration of the fibers and enzyme production profile of the fungus. The enzyme activity could not be well correlated with its corresponding fiber degradation at the same time period, as the laccase activity was lower on 60th day in all the cases while the maximum lignin degradation was recorded on 60th day of incubation. Thus the estimated amount of degraded lignin may be attribute to the cumulative effect of 60 days of fermentation while the enzyme production started earlier and showed better activity during 10 to 30 days (Figure 4.15).

Higher ligninolytic enzyme production as compared to other polysaccharide degrading enzymes, enhances the lignin degradation over other polysaccharides, which results into selective ligninolysis. It minimizes the loss in TOM as well as enhances the digestibility of the substrate. Biodelignification of straw seems to be a promising approach to overcome the problem of its lesser digestibility and lower nutritive value by enhancing the digestibility and protein content (Taniguchi et al., 2005). The present study corroborates the earlier observations, where it has been demonstrated that the improvement in nutritional quality and digestibility of feed material is not only dependent upon the fungal strain used but also on the type of substrate used (Okano et al., 2006; Chalamcherla et al., 2009).

Though selective ligninolysis is associated with IVD but some contradictory findings were also observed. During present study, D. flavida degraded lignin efficiently and enhanced protein content but was unable to enhance the IVD. Similarly, earlier study on solid-state cultivation of the white rot fungus Lentinula edodes on wheat bran demonstrated that total and insoluble dietary fibre and crude protein content increased with fungal growth while the in vitro dry matter enzyme digestibility decreased (Lena et al., 1997). In another report, about 50 % of the white rot fungal strains decreased in vitro substrate digestibility during screening experiments carried out using wheat straw as substrate (Capelari and Zadrazil, 1997). Barahona et al., 2003 reported that despite a high nitrogen content in most species, in vitro digestibility estimates were low, which was also found true in the case of D. flavida during present study.
The present study is in consonance with earlier observations on corn stover degradation by \( P. \) \textit{brevispora} which enhanced the digestibility of the substrate while \( P. \) \textit{chrysosporium} degraded maximum lignin along with a large amount of cellulose (Chen \textit{et al.}, 1995). The same study has reported that \( P. \) \textit{chrysosporium} reduced the digestibility of substrate, which is in contrast to the current findings. During the cell wall degradation study of maize, Chen \textit{et al.}, (1996) concluded that \( P. \) \textit{brevispora} exhibited stronger ability to degrade cell-wall-bound phenolic acids and suggested its better effectivity for enhancement of fibre digestibility as endorsed during present study.

### 5.3. Fungal degradation of lignocellulosic residues and associated changes in its physicochemical properties

Fungal degradation of straw brings about a variety of changes in the biological, chemical and physical properties of the substrates. During the degradation of lignocellulosic residues there was a change in its colour, aroma, pH and enhancement in total phenolic content. These changes may be because of the production of some acidic compounds in the degraded straw, which are formed during fungal degradation of lignin, cellulose or hemicellulose. Visual observation of WS showed the colour change during fermentation process, which is due to the breakdown of complex plant cell wall polymers, production of some metabolites and fungal growth on the substrate. Fungal degraded WS turned pinkish-white in colour as compared to uninoculated control. Commission Internationale de l'éclairage (CIE) colour measurements showed that yellow colour was significantly higher in degraded WS. Similarly, the redness of WS was also enhanced by all the fungi. This change in colour might be contributed by the quinone like smaller phenolic compounds, which are reported to be coloured in general and yellow or brown in specific (Murata \textit{et al.}, 2002).

The change in pH may be the result of the production of acidic compounds by the degradation of complex sugar and lignin. Studies have reported the formation of a mixture of acids including aromatic carboxylic acids during lignin degradation e.g. benzoic acid, \( p \)-methoxybenzoic acid, veratrmen acid, \( i \)-hemipinic acid, Phenoxyacetic acid, 2-methoxyphenylacetic acid, salicylic acid, trans-cinnamic acid etc. along with some \( \beta \)-keto acids, which are converted to aromatic carboxylic acids in a second oxidation step with hydrogen peroxide (Javor \textit{et al.}, 2003; Ko \textit{et al.}, 2009).
Similarly the change in aroma i.e. having slightly pungent smell in degraded straw indicates the presence of aldehydes and/or acidic compounds in the degraded straw. During earlier studies on degraded and by products of lignin, several compounds including carboxylic, aldehydes and ketone groups were identified (Maman et al., 1996) before the complete mineralization of the lignin. The metabolic pathway of the fungi plays an important role in the production of different intermediate compounds which depends upon their enzymatic activity, fungal strain and substrate. Some fungi efficiently mineralizes lignin in very short period while others take longer time and produces higher amount of smaller/monomer phenolic units (Tuomela et al., 2002). This was also evident from the TPC produced, which did not show similar enhancement in degradation of lignin during lignocellulose degradation by different fungi. This can be attributed to either complete degradation of lignin to CO₂ and H₂O, while in some fungi high TPC associated with low lignin loss indicate the production of smaller phenolic units and their further degradation to a limited extent.

A positive correlation between TPC and lignin loss (r = 0.925) showed the enhancement in smaller phenolic units during the break down of lignin. During 30 days of incubation, *P. floridensis* and *P. radiata* degraded almost similar amount of lignin but significantly higher TPC was observed in the former case. The slight difference in TPC and lignin degradation may be due to the difference in the pattern of ligninolysis, quality and quantity of smaller phenolic units formed, their solubility and further transformation as well (Yang et al., 2010). During 30 days of incubation, *P. fascicularia* caused minimum loss in lignin and thus accompanied by minimum enhancement in TPC. Due to lower TPC the antioxidant activity was also lower as compared to other fungi, though in comparison to undegraded straw, the TPC was higher and so was the antioxidant activity (Table 4.2, 4.4).

Antioxidants generally enhance different aspects of cellular and noncellular immunity. Without adequate antioxidant nutrient reserves, cellular machinery will be damaged by the free radicals, thereby reducing the effectiveness of the immune response. When antioxidant capacity is limited, the lifespan of immune cells is reduced and an infection can become established or severity of an infection can increase (Weiss, 2005). Thus, the provision of some antioxidants in the animal feed supplement might be of potential in improving the nutritional value of bioprocessed lignocellulosics as
animal feed. Straw contains about 18-24% lignin of the total biomass. Because of its heterogeneous polymerization, lignin exhibits poor radical scavenging activity. As reported by Pan et al., (2006), the lignins with more phenolic hydroxyl groups, less aliphatic hydroxyl groups, low molecular weight, and narrow polydispersity showed high antioxidant activity. Total phenolic content and antioxidant activity of WS and PS were significantly enhanced by all the fungi. TPC correlated positively with antioxidant activities; indicating the bioactive potential of phenolic compounds which neutralized the free radicals (Arora et al., 2011). Thus, the ligninolysis of lignocellulosic residues provides an added feature to the feed for the enhancement of its antioxidant potential and is in consonance with earlier observations (Pouteau et al., 2003).

Ash constitutes inorganic matter and is required in trace amounts. As ash does not contain any energy, so it would naturally lower the overall energy of the feed (Fonnesbeck et al., 1981; Frei et al., 2011). During fungal degradation of lignocellulosic residues, an increase in the ash content was observed. The straw degraded by P. chrysosporium contains a large amount of ash i.e. 22 % (ash content increased from 10.2 to 22 %) while in the straw degraded by P. brevispora had a lower ash content of 12.6 %. Ash content can be positively correlated with TOM loss ($r = 0.995$), which also indicates that the residual ash constituents were not utilized by the fungi; thus the fungus causing maximum TOM also contributes in higher ash content. Thus, the higher ash content in straw degraded by P. chrysosporium may be a factor for its lower IVD even after degrading sufficient amount of lignin. About 50 % loss in TOM and more than 20 % ash content in residual matter, both these factors does not allow the fungus to be used for processing of lignocellulosic residues to be used for animal feed production. In contrast, the ash content showed a weak positive correlation with in vitro digestibility ($r = 0.588$).

5.4. Lignocellulolytic enzyme production

The fungal lignocellulolytic enzyme system plays an important role in governing the degradation of plant cell wall fibers. This system mainly consists of cellulases, hemicellulases and ligninolytic enzymes. Phlebia spp. produce most of the lignocellulolytic enzymes and also show selective ligninolysis (Arora and Gill, 2005). Studies have reported that the use of xylanases and cellulases as feed additives can be
attributed mainly to improved ruminal fiber digestion resulting in increased digestible energy intake (Beauchemin et al., 2003).

Xylanase breaks down the hemicellulosic component of the cell wall material of lignocellulosic biomass and liberates nutrients making it freely available to the animals. It also cleaves high molecular weight arabinoxylans in animal feed. The enzyme production profile could not be correlated well with the degradation of specific polymer, which showed that fiber degradation not only depends upon the production of enzymes but also regulated by a variety of physicochemical factors. Cellulase is also an important fiber degrading enzyme that can have significant impact on improving feed utilization and animal performance. Earlier studies on different substrates like oat hulls, alfalfa and wheat straw suggested that the addition of fibrolytic enzymes (including xylanase, cellulase and glucanases) to poorly digestible feeds has the potential to enhance ruminal degradation and digestion, efficiency of feed utilization by ruminants and milk production in dairy cows (Yang et al., 2000; Beauchemin et al., 2003; Yu et al., 2005).

As reported earlier (Arora and Sandhu, 1986; Elisashvili et al., 2001), submerged conditions enhanced the laccase production as compared to solid state, while MnP was better produced during solid state fermentation. Similarly, in the present study, maximum laccase was produced at higher level of moisture though maximum ligninolysis occurred at a lower moisture level. At higher moisture level, there may be an improper mycelia adhesion to substrate’s cell wall or diffusion of the enzyme in broth medium, which might be responsible for lesser degradation of lignin hemicellulose matrix. Degradation rate of lignocellulose was better achieved under solid state conditions, which indicated that the process might require aerobic conditions and were difficult to maintain under high levels of moisture. Apparently it seems that during lignin degradation, presence of mycelia may play an important role. As evident from studies of Kurek and Odier (1990), who have shown that free exocellular lignin peroxidase produced by P. chrysosporium stimulates the mineralization of lignin, while the lignin degradation by the same enzyme seems to be more effective when it is localised on the surface of the mycelium. Further, during the present study, no direct correlation could be established between enzyme production and lignin degradation, which is in consonance with earlier observations of Levin et al., (2008), where
enzymatic activities and weight loss of the substrate did not show any significant correlation.

5.5. Degradation of straw obtained from different geographic locations

Studies suggest that fungal profile of lignin degradation and improvement in digestibility depends on the nature of lignocellulosic material and the fungal strain employed (Okano et al., 2006; Chalamcherla et al., 2009). It is like treating the different straw/substrate with the same chemical resulting in variable effect on the biochemical properties of the substrate (Vadiveloo and Fade, 2009). Studies have demonstrated that production and composition of paddy straw may also vary due to the difference in the altitude of the land as well as the season of cultivation (Williams et al., 1996), resulting in variable susceptibility of such straw to fungal degradation as observed in present study.

The ratio of plant cell wall constituents vary from plant to plant as well as in the same plant depending upon the various environmental factors, which is governed by their geographic location of cultivation (Cherney et al., 2003; Whalley et al., 2008). Lignin comprises of three main phenolic propanoid units. The ratio of these monomeric alcohols varies in lignin of different origin and affects the degradability of plant cell wall (Grabber, 2005). Studies have demonstrated that lignification in the cambial layer and early developing xylem is affected by seasonal variations (Christiernin, 2006). It is also reported that the \textit{in vitro} digestibility may differ from one to another part of the same plant (Vadiveloo, 2000). Thus, the digestibility depends upon cell wall composition of the substrate, which is further governed by a variety of factors including the climatological conditions and geographic locations. Keeping in mind the concept, both the straw samples were collected from different geographic locations of India and evaluated for their chemical analysis before and after fungal degradation.

\textit{P. floridensis} produced significantly (P < 0.05) higher laccase and resulted in good ligninolysis of PS irrespective of its zone of procurement. A maximum amount of lignin (22.8 \%) was degraded by \textit{P. radiata} in the sample collected from NWZ while it degraded a lower amount of lignin (16.4 \%) in the sample collected from NEZ (Table 4.12), reflecting a specific profile of fiber degradation depending upon the origin of substrate. \textit{P. fascicularia} degraded 21 \% of lignin in PS obtained from NWZ while it
degraded only 10 % lignin in the other two samples of PS. On the other hand, *P. brevispora* degraded almost same amount of lignin and TOM in all the PS samples obtained from geographically different zones (Table 4.5, 4.12, 4.13). It is also suggested that beside the constituents and concentration of lignin in the substrate; the holocellulose binding capacity of lignin polymer may also be responsible for the fungal behaviour towards cell wall degradation.

Lignocellulosic substrate significantly affects the fungal enzyme production. When the fungi were allowed to degrade WS of different origin, the laccase activity was higher as compared to PS degradation. In general, the fungi could degrade about 30 % lignin in WS and a lower amount (about 20 %, in general) in PS samples collected from different locations. In all the cases an increase in digestibility was also observed. From both the studies it can also be concluded that the fungal degradation pattern depends upon the biochemical composition of the substrate, which is influenced or governed by their geographic and climatological conditions.

5.6. Fungal degradation of lignocellulosic residues in the presence of nitrogen rich supplements

Production of ligninocellulolytic enzymes and ligninolysis is affected by various physiological conditions including moisture level, temperature, pH, presence of chemical compounds etc. These parameters are generally taken in account for the enhancement or optimization of enzyme production and/or ligninolysis. During solid state fermentation of straw, enhancement in lignocellulolytic enzyme production, digestibility and nutritive quality using some white rot fungi and nitrogenous supplements have been studied earlier (Zadrazil and Brunnert, 1980; Mikiashvili *et al.*, 2006). But still a lot needs to be done to optimize the various parameters to get better results for enhancement in digestibility and nutritive value of agro residues. As mentioned earlier, on the basis of results obtained from degradation study of WS and PS obtained from different geographic locations, two fungi *P. brevispora* and *P. floridensis* were selected for further degradation because of their better selectivity towards ligninolysis and enhancement in IVD. Further studies were conducted using straw samples collected from NWZ because of its higher susceptibility and abundant availability. The straw samples were degraded by the selected fungi in the presence of different nitrogen rich supplements under SSF conditions.
Several contradictory reports are available on the effect of supplements on enzyme production. In most of the cases, production of different enzymes was enhanced by the addition of nitrogen sources. During earlier experiments (Arora and Gill, 2005), \textit{P. floridensis} produced higher amount of laccase and MnP in nitrogen rich mineral salt broth medium. However, lignin peroxidase was produced only in nitrogen-limited medium. On the other hand Kachlishvili \textit{et al.}, (2005) reported that neither the laccase nor the MnP yield was significantly affected by the additional nitrogen source during SSF of wheat straw by different white rot fungi, while in some cases hydrolytic enzymes and laccase production increased during fermentation. So, it was concluded that SSF of wheat straw and beech tree leaves, was strain and substrate dependent.

During a previous study with \textit{P. ostreatus}, it was reported that as compared to organic compounds, enzyme activity decreased with supplementation of inorganic nitrogen sources and concluded that the effect of carbon and nitrogen sources depends on the fungal strain and nature of the compound tested (Mikiashvili \textit{et al.}, 2006). Similar observations were made during the present study, where the presence of different compounds showed difference in lignocellulosic degradation and enzyme production depending upon the strain employed.

The use of supplements may be worth to improve the performance of the organism in terms of lower TOM loss, enhanced ligninolysis and IVD of WS. Inorganic and organic nitrogen rich supplements were used for the purpose, as the carbon nitrogen ratio is an important factor for the enzymatic regulation and lignocellulose degradation by fungi (Zadrazil and Burnnert, 1980; Shrivastava \textit{et al.}, 2011). As compared to inorganic supplements, the overall enzyme production and fiber loss was higher with organic supplements during the present study. Organic supplements contain sufficient reduced carbon and nitrogen and are rich in amino acids and simple sugars, which might be responsible for the better enzymes production, fungal growth and the degradation of fibers (Hatvani \textit{et al.}, 2003; Reid, 1983).

All the inorganic supplements significantly (P < 0.05) minimized TOM loss in WS while only \(\text{NH}_4\text{Cl}\) accelerated the ligninolysis and IVD as compared to control. On the other hand, in PS no such distinctive loss in TOM was observed during its degradation by \textit{P. brevispora} or \textit{P. floridensis}. Among organic supplements malt extract
during WS degradation, while peptone and soya bean meal during PS degradation, enhanced the IVD up to a maximum extent (Table 4.14-4.17).

To study the effect of both of these supplements (one organic and one inorganic) individually and collectively, a RSM based experiment was designed to optimize their concentration along with moisture content. The use of statistical RSM design for the optimization purposes allow to study the impact of single factor as well as the interactions with other factors. The combination of these variables may also be used for optimization of more than one parameter, which was found to be useful in the present study. RSM is a widely used tool and was used earlier to enhance the production of different lignocellulolytic enzymes in SSF by using a wood-based solid medium supplemented with malt extract (Levin et al., 2008). The degradation of lignocellulosics and the digestibility is the phenomenon governed by a variety of factors. However, as it is not practically possible to handle all the parameters simultaneously, thus some of the parameters were considered and optimized successfully during the present study.

During WS degradation by *P. brevispora*, a higher concentration of malt extract supported better fungal growth and higher degradation of lignocellulosic biomass along with the enhancement in IVD while, NH$_4$Cl supported a better break down of lignin polymer accompanied by apparently less fungal growth and lower degradation of biomass. Malt extract itself is highly digestible by ruminants and used by the fungi for their initial growth on a complex substrate like WS. Although malt extract supported maximum enhancement in the digestibility of WS, it also supported a maximum loss in TOM (18.8 %), which reflects its limitation vis a vis NH$_4$Cl as selective ligninolytic action is very important in such studies and it was more pronounced in the presence of NH$_4$Cl where only 12 % TOM was lost with 25.5 % loss in lignin (Table 4.18). The organism clearly showed selective and statistically significant (P < 0.05) ligninolytic behaviour under such conditions.

On the basis of RSM studies on WS degradation by *P. floridensis*, variable optimal conditions were observed for different responses i.e. enzymes production, fibers degradation and IVD. The optimal conditions for the enhancement in *in vitro* digestibility, were also favourable for the maximum production of both the polysaccharide degrading enzymes such as CMCase and Xylanase (Table 4.20). Malt extract acts as a good source of nutrition and easily digestible by the ruminants thus
responsible for enhancement in the digestibility of feed. However, the use of high concentration of malt extract may not be economic for the production of animal feed. On the other hand, simply for the enzyme production, the media and conditions used in the present study may prove to be much economic and easier as compared to most of the previously optimized media which requires more expensive chemicals (Cai et al., 1999).

Increase in moisture content significantly enhanced the laccase activity. Similar results were also reported by Elisashvili et al., (2008) where more laccase was produced during submerged than solid state fermentation. However, the lignin degradation requires lesser moisture content as it is a strictly oxidative process and thus requires oxygen (Villas-Bo’as et al., 2002), which is difficult to maintain under submerged or high moisture levels. Further, it might be possible that under SSF conditions the enzyme is more intimately adsorbed on to the surface thus, affecting better degradation. RSM studies clearly reflected that higher nitrogenous supplements did not accelerate the production of all the enzymes and lignocellulosic degradation. Simultaneous addition of both the nitrogenous supplements (NH₄Cl and malt extract) suppressed the degradation of all the fibers as compared to addition of any single supplement. Earlier studies have also reported that ligninolytic activity is indeed suppressed by excess nitrogen, while growth-limiting nitrogen levels stimulate the extent of lignin degradation in different plant residues (Kachlishvili et al., 2005; Commanday and Macy, 1985).

During present study, addition of nitrogen sources enhanced the enzyme production alongwith fiber degradation, but the higher concentration of both the nitrogen sources collectively was found to be suppressive. These findings are in the consonance with previous observations (Yang et al., 1980; Hatvani, 2003). In the literature, there are contradictory reports on the effects of nitrogen sources on ligninolytic enzyme production. Lentinula edodes produced MnP and laccase, when grown in a defined medium with glucose as sole carbon source. MnP production was suppressed by nitrogen while highest amount of laccase was produced when the fungus was grown under high nitrogen conditions (Buswell et al., 1995). In another study, MnP activity was higher in Pleurotus sajor-caju, supplemented with higher nutrient nitrogen concentration. Conversely, laccase production was not influenced by nitrogen levels under the growth conditions (Fu et al., 1997).

5.7. Up scaling the degradation process
The up scaling of SSF of both the lignocellulosic residues (WS and PS) from 5g to 200g was conducted successfully under optimized conditions. Solid state fermentation is an advantageous method to degrade lignin and improve the digestibility of lignocellulosics. Fungi grown under these conditions perform better ligninolysis and the addition of fungal mycelium contributes to the total protein content of the feed (Fazaeli, 2007). The results also demonstrated the increase in amino acid content, TPC and antioxidant property in fungal degraded straw, thus reflecting an enhancement in nutritional qualities. Enhancement in antioxidant activities have been earlier reported during the solid state fermentation of some agricultural residues by *Rhizopus stolonifer*. Thus, the strategy may be used for upgrading low quality agro wastes to develop healthy animal feed supplements (Lateef et al., 2008). The production and extraction of bioactive phenolic compounds from natural sources by SSF using fungal sources has also been discussed in a recent review (Martins et al., 2011).

During microbial processes for conversion of lignocellulosic wastes into feed, at least one of three objectives must be reached: an increase in the digestibility of the lignocellulosic material, an increase in the protein level and an improvement in the dry product palatability, although the last factor can be easily improved by ensiling or mixing the substrate with other more palatable foods (Kamra and Zadraz’il, 1988). As evident from the present study, first two important objectives were achieved successfully.

Figure 5.3 shows the increase in IVD before and after optimization with respect to control i.e. undegraded straw. During WS degradation, *P. brevispora* caused more than 15 % loss in TOM with concomitant enhancement in IVD from 17.2 to 28.7 % (62 % increase in IVD) after 30 days of incubation period. However during scale up experiment, almost similar enhancement in IVD (about 60 %) was achieved during 20 days of incubation at the cost of 10-11 % loss in TOM (Table 4.2, 4.26), under optimized conditions. Similarly in the case of PS, *P. floridensis* degraded about 11 % of biomass and enhanced the digestibility from 18.5 to 23.2 (25 % increase in IVD) during 60 days of incubation period. During 20 days of incubation under optimized scaling up experiment, upto 25.8 % (40 % increase in IVD) was achieved at the cost of only 6 % loss in TOM (Table 4.5, 4.29). Thus the process seems to be effective and efficient for the bioprocessing of lignocellulosic residues.
Figure 5.3 Percentage enhancements in IVD during 20 days of degradation before and after optimization with respect to control

The fermented straw is a mixture of straw and fungal biomass. Estimation of the fungal biomass in fermented straw is very difficult as the experiment was performed under solid state, which makes it difficult to separate the fungus from the straw. Roche et al., (1993) have proposed a method to measure the fungal biomass by estimating the chitin content. The idea was adopted to calculate the fungal biomass present in the degraded straw and found to be helpful, which revealed that finally the feed contained about 5.5-6.5% of fungal biomass. All the fungi were demonstrated to be non cytotoxic and non mutagenic (Arora et al., 2011), thus a safe, nutritional and easily digestible feed may be provided after bioprocessing of straw.

Several studies have successfully demonstrated the important use of these cell free ligninolytic enzymes (crude cell free extracts) in the decolourization of dyes present in some textile effluents. Beside this several biotechnological applications of ligninolytic enzymes have been discussed (Trupkin et al., 2003; Chander and Arora, 2007). Solid state fermentation of soy and wheat bran has been successfully used for the production of different lignocellulolytic enzymes (Papinutti and Forchiassin, 2007), which can be used in several industries after purification or as such. In present study the production of these enzymes have been demonstrated successfully using an economic and easily available raw material (agricultural residues) under solid state conditions, which can
also be harvested as by product during fermentation process and used for the various industrial purposes.

In the light of foregoing discussion the present study can be concluded as under:

All the tested white rot fungi efficiently degraded the lignin and enhanced the \textit{in vitro} digestibility of straw; except \textit{D. flavida}, which could not enhance the digestibility. \textit{Phlebia} spp. enhanced the \textit{in vitro} digestibility almost to a similar level as compared to widely studied fungus \textit{P. chrysosporium} and demonstrated much lower loss in total organic matter as compared to \textit{P. chrysosporium}.

Biochemical constituents of straw varied with respect to their climatologically different geographical locations. The profile of fungal degradation of lignocellulose and enzyme production depends upon straw constituents, which was governed by climatological conditions.

\textit{P. brevispora} and \textit{P. floridensis} were the best organisms to provide a practically promising approach in selective ligninolysis and enhancement of \textit{in vitro} digestibility of wheat straw and paddy straw, respectively as they were efficient to degrade the straw irrespective of their region of procurement.

As the bioprocessing of agricultural residues for animal feed is a vital system in the emerging field of feed biotechnology, white rot fungi may be used as efficient organisms for lignocellulolytic enzyme production, selective lignin degradation and resultant enhancement in \textit{in vitro} digestibility. This study of fungal degradation of agro residues describes an ecofriendly system for the development of processes and products having economical and social relevance to animals and humankind. As the ligninolytic enzymes produced can be used for various industries.

It can be further concluded that the selected strains may prove useful in value-addition of the lignocellulosic wastes, towards their utilization as healthy feed supplements, particularly in protein content and antioxidant activities; thereby reducing the cost of producing value added feeds for the ruminants.