Discussion
The scarcity of green fodder to sustain present level of livestock in our country is of a big challenge. The agricultural crop residues are meeting out the fodder demand to a larger extent. But the low digestibility, protein content, poor palatability and high lignin content of available agroresidues make them inadequate as feed. Moreover, majority of the poor nutrient availability of plant material could be attributed to the lignification and crystallinity of cellulose. To remove lignin from these plant material using chemical, mechanical or thermal processes are either not environmentally benign or facing cost related issues. Therefore using biological pretreatment, especially with the help of white rot fungi to improve the digestibility and nutrient quality of these residues seem to be a viable option (Zadrazil and Brunnert, 1980; Yadav and Tripathi 1991; Zadrazil and Punia, 1995; Abdullah et al., 2004; Kristensen et al., 2008; Shrivastava et al., 2011, 2012; Tuyen et al. 2013). However, most of the basidiomycetous fungi (white-rots) are reported to degrade lignin, cellulose and hemicellulose simultaneously. The important alternatives to make these processes successful are (i) the requirement of longer incubation period by these fungi and (ii) producing an inoculum (fungal seed) with high self life at larger scale.

This necessitates to search for some more competitive and selectively lignin degrading fungi and optimize the fermentation process for maximum lignin degradation and least utilization of carbohydrates (Basu et al., 2002; Okano et al., 2005, 2006; Shrivastava et al., 2011).

The present study was undertaken with the aim to find a suitable selective lignin degrading fungus and optimizing the inoculum development process. Solid state fermentation (SSF) of wheat straw was carried out using pellet inoculum and the fermented feed was evaluated in rat model for its toxicological status and nutrient digestibility. The fermented feed was then also tested using in vitro technique to assess its nutritional status and the process of animal feed development was statistically optimized to maximize lignin degradation and minimize cellulose and hemicellulose consumption.
Finally, the process of animal feed development was scaled up and feed was evaluated in vivo (in calves) and digestibility of nutrients was studied.

**Study the diversity of basidiomycetous fungi**

A fungus suitable for animal feed development is, which can colonize the substrate fast, degrade lignin selectively. Keeping this in view, fungal samples were collected from various habitats and geographic locations, majorly growing on wood and plant litter. The samples were carefully separated from their substrates and brought back to lab and cultured to purity through successive sub culturing. In addition some well known lignin degrading fungi such as *Pycnoporus cinnabrinus*, *Pleurotus ostreatus*, *Pleurotus sajor caju*, *Trametes versicolor* and *Phanerochaete chrysosporium* were also included in the study. *Crinipellis* sp. RCK-1 and *Ganoderma* sp. rckk02 (our own lab cultures) were also studied along with them. Among 18 fungi studied, *Crinipellis* sp. RCK-1 and *Ganoderma* sp. rckk02 were found to be fastest growing followed by *P. cinnabrinus*, *P. chrysosporium*, *P. ostreatus* and *T. versicolor*.

The ability of various fungi to colonize wheat straw was studied to find suitable organism for SSF of wheat straw to transform it in to animal feed. The growth study revealed that, *Crinipellis* sp. RCK-1 and *Ganoderma* sp. rckk02 were found to be fastest colonizers of the wheat straw followed by *P. cinnabrinus*, *P.chrysosporium*, *P. ostreatus* and *T. versicolor*. Comparatively these fungi grew slowly on wheat straw then on MEA medium. Mycelial growth of fungi is largely reported to vary with the substrate characteristics, temperature and the presence of supplements in the growth medium (Eichlerova et al., 2000; Zervakis et al., 2001; Philippoussis et al., 2001; Jonathen et al., 2008). The growth rate of some fungi may differ upon growing them on different substrates or even within different strains of same fungi (Eichlerova et al., 2000; Jonathen et al., 2008). *Crinipellis* sp. RCK-1 and *Ganoderma* sp. rckk02, exhibited faster growth on wheat straw i.e. 8.9 cm on 5th and 7th day respectively, when compared with growth of *Pleurotus tuber-regium* on 4 different Nigerain wood waste, *Terminalia superba*, *Mansonia altissima*, *Holoptelia grandis*, and *Milicia excelas* (6.2, 8.4, 6.7 and 5.3 cm, respectively) till 7th day. Based on the growth and substrate
colonization, six fungi (*Crinipellis* sp. RCK-1, *Ganoderma* sp. rckk02, *P. cinnabrinus*, *P. chrysosporium*, *P. ostreatus* and *T. versicolor*) were selected for further study.

SSF of wheat straw was carried out using six fungi to check their ligninolytic potential, while degrading minimum of cellulose. *Crinipellis* sp. RCK-1 and *Ganoderma* sp. rckk02 degraded highest amount of lignin (22.57 and 27.27 %, respectively) on 10th day of incubation with minimum loss of cellulose (~19%). C/L ratio was increased from 3.66 in unfermented straw (control) to a maximum level of 3.85 and 4.08 in case of *Crinipellis* sp. RCK-1 and *Ganoderma* sp. rckk02 until 15th and 10th day respectively, which revealed these fungi have capacity to degrade lignin at faster rate than cellulose. The sole objective of this screening was to find a suitable white rot fungal strain which can selectively take out lignin without touching much of cellulose (the carbohydrate content which serves as energy source for ruminants). Similar emphasis has been given in various earlier reports pertaining to animal feed development (Basu et al., 2002; Okano et al., 2005, 2006; Shrivastava et al., 2012). Moreover, rest of the fungi such as *P. cinnabrinus*, *P. chrysosporium*, *P. ostreatus* and *T. versicolor* were omitted from further experiments due to their lower ligninolytic potential, and higher carbohydrate consumption rate as evident from their decreasing C/L ratio throughout the fermentation.

Similarly Capelari and Zadrazil (1997) screened 72 Brazilian fungi related to *Agrocybe*, *Antrodiella*, *Auricularia*, *Coriolopsis*, *Cymatoderma*, *Fomitopsis*, *Ganoderma*, *Gerronema*, *Gloeophyllum*, *Gymnopilus*, *Irpex*, *Lentinus*, *Melanoporia*, *Oligoporus*, *Oudemansiella*, *Panaeolus*, *Peniophora*, *Phellinus*, *Pleurotus*, *Psathyrella*, *Psilocybe*, *Pycnoporus*, *Rigidoporus*, *Schizophyllum*, *Trametes*, *Trichaptum* and *Tyyromyces* genus and out of them 22 decomposed more than 50 % of lignin. The highest degradation of lignin was observed with *Lentinus crinitus* (80 %, 60 d) and the highest increase in *in vitro* substrate digestibility was caused by *Peniophora utriculosa* (35.9 % after 30 day of SSF). This study articulated that fungi collected from even similar habitats might have differential growth characteristics and capability to degrade lignin. To the best of our knowledge based on literature survey, *Pleurotus* sp. has been found to be largely used
fungus for animal feed development studies, chiefly due to its non toxic nature and edibility, followed by other fungi like *P. chrysosporium*, *Ceriporiopsis subvermispora*, *Coprinus fimetarius* and *Lentinus edodes*. Moyson and verachtert (1991), studied capability of *Pleurotus pulmonarius*, *pleurotus sajor caju* and *Lentinus edodes* to improve digestibility of wheat straw through selective lignin degradation. These fungi were observed to degrade 61.98, 62.81 and 38% of lignin at the cost of 7.42, 12.79 and 22.51% degradation of cellulose within 12 weeks of fermentation. However, in addition to the very long incubation time the degradation of hemicellulose was also quite high as 58, 59.41 and 43 %, respectively, for *P. pulmonarius*, *P. sajor caju* and *L. edodes*. In our study, *P. ostreatus* was observed to degrade almost similar amount of lignin, cellulose and hemicellulose (34, 35 and 36 %, respectively), within 15 day of fermentation, which did not qualify it as a promising fungus for animal feed development. Similarly, *T. versicolor* degraded higher amount of cellulose and hemicellulose than lignin within 15th day of SSF i.e. 19.8, 20.01 and 11.01 %, respectively. *P. ostreatus* has been found to degrade variable amount of lignin (L) cellulose (C) and hemicellulose (H) under various experimental conditions and using wheat straw viz. ~20 % in L, C within 15th day (Adamovic et al., 1998); 11.97 % L, 16.42 % C (Akinfemi et al., 2010); 31.09 % L, 29.03 %C and 33.43 % H (Shrivastava et al., 2011); 50.9 % L, 20.1 % C and 56.6 of H within 6 weeks of SSF (Tuyen et al., 2013). *T. versicolor* has also exhibited differential ability to degrade cell wall components with different substrates viz. 8.93 % L, 12.92 %C and 21.34 % of H of wheat straw within 15 day of SSF (Shrivastava et al., 2011); 4.5-35 % L, 0.28-11.07 % C and 2.14- 21.18% of H, within 21 days of SSF of corn stover (Zhu et al., 2011).

In the present work, *P. chrysosporium* and *P. cinnabarinus*, were observed to degrade higher amounts of cellulose and hemicellulose compared to lignin and this has been reported in various earlier studies, which is undesirable for animal feed development (Agosin et al., 1985; Arora et al., 2002; Basu et al., 2002; Sharma and Arora, 2010a). *P. cinnabarinus* has not been studied much for animal feed development earlier and largely studied for production, purification of laccase enzymes (Agosin et al., 1985; Eggert et al., 1996; Sharma et al., 2012). These findings revealed that all
these fungi were able to degrade lignin at later stages of incubation but at initial stage these fungi needs some easily available or utilizable carbon sources for their own growth, therby limiting their availability for rumen microflora (Zadrazil, 1980; Sharma and Arora, 2010b).

Extracellular plant fiber degrading enzymes have been reported to have a significant role in degradation of plant material and increasing the availability of nutrients in rumen and addition of these fibrolytic enzymes in poorly digestible feed has been reported (Beauchemin et al., 2003; Sharma and Arora, 2013). Xylanase breaks the hemicellulosic content, and cellulase is responsible for degradation or modification of cellulose, eventually liberating nutrients in rumen of cattle. In the present work, maximum laccase and xylanase activities were observed on 10th day of incubation in case of Crinipellis sp. RCK-1, Ganoderma sp. rckk02, P. ostreatus and T. versicolor. P. chrysosporium did not produce detectable amount of laccase however, it was observed to be a potent producer of xylanase (142 IU/g) on 15th day of incubation. While, P. cinnabarinus was observed to be a good producer of laccase and poor producer of xylanase enzyme (196 and 9.64 IU/g, respectively till 15th day). All fungi studied here exhibited minimal production of cellulase and its production peaked on 10th day of incubation in case of Crinipellis sp. RCK-1, Ganoderma sp. rckk02 and P. chrysosporium and on 15th day for rest of the fungi. However, among the fungi tested here, only P. chrysosporium was found to produce fair amount of cellulase (> 1.7 IU/g) till 10th day of SSF. While, Ganoderma sp. rckk02, P. chrysosporium and T. versicolor produced 0.97, 0.43 and 0.91 IU/g of cellulase on 5, 15 and 10th day respectively. Moreover, Crinipellis sp. RCK-1, P. ostreatus and P. cinnabarinus also did not produce any detectable amount of LiP.

Similarly, P. chrysosporium produced fair amount of MnP on 10th day of incubation (7.01 IU/g), while, low titer (4.34 and 1.34 IU/g) of MnP was observed in case of in case of Crinipellis sp. RCK-1, Ganoderma sp. rckk02 on 10th day. Among all the fungi tested T. versicolor was found least producer of MnP (1.34 IU/g on 15th day).

Moreover, the enzyme production profile could not be correlated well with the degradation of respective components except the low titer of
cellulase, which might have helped in preserving cellulose content in *Crinipellis* sp. RCK-1 and *Ganoderma* sp. rckk02 fermented wheat straw. Almost similar titers of cellulase and xylanase were obtained, when wheat and rice straw were fermented using *Pleurotus sajor- caju* under SSF conditions for animal feed development (Bisaria et al., 1997). The fungus produced maximum cellulase 1.7 and 2.4 (IU/g) and approximately 8-12 (IU/g) of xylanase after 20 days, while growing on rice and wheat straw, respectively. The production maxima of LiP and Mnp by *P. chrysosporium* (0.43 and 7.01 IU/g, respectively) and *T. versicolor* (0.91 and 1.35 IU/g, respectively) in present study were well in accordance to the level of these enzymes reported earlier during SSF of wheat straw (Arora et al., 2002; Huang et al., 2010). However, higher level of laccase xylanase and MnP have also been reported by *P. ostreatus* and *T. versicolor* during SSF of wheat straw for cattle feed development under optimized conditions (Shrivastava et al., 2011). In a similar study during SSF of wheat straw for animal feed development by *P. floridensis*, *P. radiata*, *C. subvermispora* and *P. brevispora*, production of laccase peaked at 30th day of incubation (Sharma and Arora, 2010a). *P. chrysosporium* was also observed to be highest producer of cellalase and xylanase and did not produce laccse as has been observed in our study (Sharma and Arora, 2010a). Various studies indicated that the enzyme production profile can not be correlated well with the degradation of specific plant cell wall polymer, which shows that fiber degradation not only depends upon the production of enzymes but also regulated by a variety of physicochemical factors (Sharma and Arora, 2010a; Shrivastava et al., 2011).

**Developing fungal inoculum**

Among various types of fungal incula tested for carrying out SSF of wheat straw, fungal pellets colonized wheat straw more efficiently and rapidly. Agroresidues and wood based inocula faces the major problem of sustainability while, grain based inocula required higher cost inputs and were prone to contamination. When fungal discs used as inoculum were also not found suitable due to its improper distribution within the substrate. Various types of incula have been used by many workers to carry out SSF of different substrates and variable efficiency of fungal colonization and
degradation capability have been observed. Majorly, mycelial discs (Tripathi and Yadav, 1992; Eichlerova et al., 2000; Okano et al., 2005; Jonathen et al., 2008; Akinfemi et al., 2010; Neifar et al., 2013), fungal spore suspension (Penaloza et al, 1985; Agosin and Odier, 1985; Villas-Boas et al., 2003; Huang et al., 2010), mycelial homogenate (Tripathi and Yadav, 1992; Jalc et al., 1996, 1999; Okano et al., 2006; Li et al., 2008), grain spawn (Moyson and Verachtert, 1991; Tripathi and Yadav, 1992; Adamovic et al., 1998; Philippoussis et al., 2001; Tripathi et al., 2008; Tuyen et al., 2012), fungal mat (Tanaka et al., 2009) and fungal pellets (Basu et al., 2002; Shrivastava et al., 2011, 2012), have been used to carry out SSF. Use of fungal spore suspension has also been discouraged due to the long lag period, resulting from spore germination time and enzyme induction (Tengerdy and Szakacs, 2003).

According to Basu et al. (2002) inoculum type/seed culture has a major role in achieving high level of lignin degradation under SSF. They had prioritized the inoculum/seed culture in the form of individual and separated pellets in comparison to mycelia, for uniform inoculation of moist wheat straw substrate. The fungal pellets as inoculum has been reported to be advantageous as compared to filamentous inoculum in obtaining decreased viscosity, desirable mixing, better mass and oxygen transfer into the biomass and lower energy consumption for aeration and agitation (Suijdam et al. 1980; Liao et al. 2007). Thus use of fungal pellet inoculum might have improved the colonization and biodegradation of wheat straw. According to Suijdam et al. (1980) fungal growth in pellet form has been found a favorable alternative, which benefits most of the fungal fermentations through repeated-batch fungal fermentation possible and improving the culture rheology. However, major emphasis has been given on statistical experimental designs to be applied to find optimal conditions for fungal pellet formation due to the complication of effects of various factors on fungal morphology (Liao et al., 2007).

During one factor at a time method (OFAT) followed by Central Composite Design of RSM to improve fungal pellet production, primary culture concentration, Ca (NO₃)₂. 4H₂O and malt extract were found most influencing factor in case of Crinipellis sp. RCK-1. Whereas, agitation, malt
extract and concentration of Ca (NO$_3$)$_2$. 4H$_2$O were the most crucial factor in maximizing the pellet biomass development by *Ganoderma* sp. rckk02. Moreover, the pH of medium has also been reported very important for various fungi to form pellets and it has been found that pH can change the surface properties of fungi and influencing the pellet formation, and different strains have different sensitivity to pH (Metz and Kossen, 1977; Liao et al. 2007). However, pH of medium was not observed to influence much the fungal pellet production in present study, which could be due to lesser sensitivity of fungi used for pH and buffer strength of MEB used. Inoculum size or primary culture (in our study) is generally taken of great importance to the process of fungal pellet formation, because majorly it is the interaction of hyphae, which is considered as the main force in forming clumps (Liao et al., 2007). Thus, an optimum primary culture is important factor in producing maximum fungal pellets because in the early stage of growth, the higher inoculum size promotes more interaction with the hyphae to form pellets. On the contrary, excessive primary culture causes to form clump of hyphae instead of pellets, thus, optimum inoculum concentrations are crucial for pellet production and varies from strain to strain (Foster, 1949; Metz and Kossen, 1977, Znidarsic and Pavko, 2001; Grimm et al. 2005). MEB is largely used as a medium for the growth of white rot fungi largely due to the availability of complete pool of amino acids as evident from earlier studies on *Polyporus versicolor*, *P. cinnabarinus* and *C. bulleri* (Sandhu and Arora, 1984; Dhawan and Kuhad, 2003; Dhawan et al., 2005). The type of nitrogenous compounds also have been reported to have a significant effect on fungal pellet formation (Pirt and Callow, 1959). Dhawan and Kuhad (2004) also have reported calcium nitrate among the various inorganic sources to be important in enhancing growth and enzyme production from bird’s nest fungus, *Cyathus bulleri*. As has been observed in our study during fungal pellet formation, calcium nitrate might have supplied Ca$^{2+}$ ions, which are usually recognized to induce mycelial aggregation during fungal growth (Jackson and Heath, 1993). It has also been shown by Liao et al. (2007) that media with calcium carbonate produced smoother and larger pellets than those without calcium carbonate.
Availability of nutrients during submerged fermentation (SmF) is majorly depend on agitation rate, which is responsible for mixing of all the nutrients. Agitation provides better availability and adsorption area which ultimately becomes crucial in determining the contact time (Samaniuk et al., 2011). Lesser fungal biomass production at lower agitation may be attributed to the improper oxygen supply, which subsequently decreased the specific oxygen uptake (Shupe and Liu, 2012).

Finally a total increase of 52.5 and 77.68 % in fungal pellet biomass (g/L) was achieved through statistical optimization for *Crinipellis* sp. RCK-1 and *Ganoderma* sp. rckk02, respectively.

**Fermentative production of animal feed**

*Crinipellis* sp. RCK-1 and *Ganoderma* sp. rckk02, both degraded wheat straw more efficiently when fungal pellet were used as inoculum to carry out SSF. Compared to initial screening experiments, *Crinipellis* sp. RCK-1 degraded almost similar amount of lignin and hemicellulose but appreciably lower amount of cellulose (i.e. 15.92%), within 15 days of fermentation. Whereas, *Ganoderma* sp. rckk02 degraded higher lignin i.e. 11.74% even on 5th day of fermentation compared to 6.17% lignin degradation under un-optimized conditions. However, it simultaneously degraded higher amount of cellulose along with lignin, which could be due to more efficient colonization by fungal pellets and higher dry matter loss. Both the fungi achieved maximum efficiency of SSF (ESSF) on 10th day largely due to comparatively higher degradation of lignin than cellulose on 10th day. Percent efficiency of SSF was calculated for SSF of wheat straw using *Pleurotus pulmonarius, Pleurotus sajor caju* and *Lentinus edodes* (Moyson and Verachtert, 1991). In comparison to present study these fungi exhibited almost double ESSF, but they took too long (20 weeks) in obtaining that. In a very extensive study, among 72 Brazilian species and strains of various white rot fungi only 22 decomposed more than 50 % of lignin and highest degradation of lignin was observed with *Lentinus crinitus* (80 %,) after 60 days of SSF and highest increase in IVDMD (35.9 % increase) was obtained by *Peniophara utriculosa* after 30 day of SSF (Capelari and Zadrazil 1997).
Similarly, various fungi such as *Pleurotus* sp., *Trametes versicolor*, *Ceriporiopsis subvermispora*, *Ganoderma* sp., *Bjenkandera adusta*, *Lentinus edodes* and *Phlebia brevispora* have been studied for bioconversion of wheat straw to digestible animal feed (Shrivastava et al., 2011, 2012; Tuyen et al., 2012; Arora et al., 2011; Arora and Sharma 2011). But majority of them were observed to take longer time in degrading either similar or higher amount of lignin compared to *Crinipellis* sp. RCK-1. Prolonged incubation periods have already been reported to be associated with simultaneous degradation of cellulose and hemicellulose eventually causing an undesirable decrease in dry matter digestibility of the fermented substrate. Hence, like various other reports present study also advocated carrying out the SSF in a shorter period to prevent much of the cellulose and hemicellulose losses (Tripathi and Yadav 1992; Chen et al., 1995; Basu et al., 2002; Okano et al., 2005; Shrivastava et al., 2011). Moreover, the hemicellulose content, which was observed to be degraded at a slightly higher rate in present study, is known to play only a minor role in supplying nutrients in the cattle rumen (Tuyen et al., 2012). Statistical analysis revealed that there was a significant negative correlation ($R^2 = -0.972$) between amount of lignin present in the substrate and % efficiency of SSF in case of *Crinipellis* sp. RCK-1. Moreover, it was negatively correlated too for *Ganoderma* sp. rckk02, but was not significant (Fig. 5.1).

Both the fungi when grown on wheat straw under SSF produced lignocellulolytic enzymes during earlier days of incubation. Maximum laccase production was observed on 7th day of incubation for both the fungi (112 and 214 IU/g for *Crinipellis* sp. RCK-1 and *Ganoderma* sp. rckk02, respectively). Production of xylanase also followed the similar trend as laccase and cellulase production peaked on 9th day in case of *Crinipellis* sp. RCK-1, which might be responsible for higher degradation hemicellulose and cellulose content on 10th day. The sustained production of xylanase and cellulase could be the reason for continuous degradation of cellulose and hemicellulose till 15th day.
However, the statistical analysis revealed a very weak correlation between laccase, xylanase and cellulase and the degradation of respective cell wall components (Fig. 5.2). Among these the maximum correlation coefficient value was obtained for hemicellulose degradation and xylanase production ($R^2 = 0.622$) followed by laccase production and lignin degradation ($R^2 = 0.4826$) and cellulase production and cellulose degradation ($R^2 = 0.0019$) in case of *Crinipellis* sp. RCK-1. Comparatively, a significantly weaker correlation (<0.1) was observed in case of *Ganoderma* sp. rckk02, when analyzed between laccase, xylanase and cellulase and the degradation of respective cell wall components. Present findings are very much in accordance with the earlier hypothesis that the enzyme production profile can not be correlated with the degradation of specific polymer, which shows that fiber degradation not only depends upon the production of enzymes but also regulated by a variety of physicochemical factors (Sharma and Arora, 2010a; Shrivastava et al., 2011). Profile of lignocellulolytic enzymes have been studied in detail by various workers to get a better insight in to substrate degradation and its subsequent application in development of animal feed development. However none of this study till date has been able to predict the magnitude of substrate degradation base on enzyme production profile (Arora et al., 2002; Bisaria et al., 1997; Li et al., 2008; Tanaka et al., 2009; Huang et al., 2010; Shrivastava et al., 2011; Akinyele et al., 2011; Zhu et al., 2011; Neifar et al., 2013).
Fig. 5.2. Correlation analysis of production of lignocellulolytic enzymes and degradation of cell wall components (A-C) **Crinipellis** sp. RCK-1 (D-F) **Ganoderma** sp. rckk02
Nutritional and toxicological analysis of fermented feed

Crinipellis sp. RCK-1 and Ganoderma sp. rckk02 used in the present study have been found to degrade comparatively more lignin than cellulose, which qualify them to be used for upgrading the animal feed. The fungal fermented feeds have been evaluated for their nutritional and toxicological status in male rats as model system. The proximate analysis of composite diets revealed that all the nutrients like organic matter (OM), ether extract (EE), crude fiber (CF), nitrogen free extractive (NFE), total carbohydrate (T-CHO) and ash varied marginally among them, however, EE content of fungal fermented diets was considerably improved, which could be attributed to value addition to diet by fungal fermentation. The observed increase in crude protein and EE could have provided energy in terms of more fermentable carbohydrates and increased digestibility of fermented diets (Shrivastava et al., 2011). Similar findings are reported by Akinfemi et al. (2009), where increase in digestibility of feedstuff was attributed to the breakdown of CF and acid detergent fiber (ADF) content and increase in crude protein (CP) content. The improved dry matter intake (DMI) in Ganoderma sp. rckk02 fermented diet (T3) and Crinipellis sp. RCK-1 diet (T4) was probably responsible for higher nutrient availability. Contrary to this, diet fermented with P. cinnabarinus (T2) exhibited lower (P<0.05) DMI, consequently causing poor nutrient digestibility. The diets containing unfermented wheat straw (T1) or fermented with Ganoderma sp. rckk02 (T3) and Crinipellis sp. RCK-1 (T4) were found to be free or had negligible amount (< 3 ppb) of various mycotoxins i.e. aflatoxin B1, B2, G1 & G2. The observed levels of these mycotoxins were far below the permissible levels (20 ppb) in the feeds as per the standards of diet for immature animals by U.S. Food and Drug Administration (USFDA) (Ref. FDA/ORG Compliance Policy Guides (CPG) 7126.33, Sec 683.100) and poultry by Bureau of Indian Standards (BIS).

Biochemical studies of the blood samples showed normal level of stress enzymes in the rats fed on either Ganoderma sp. rckk02 fermented diet (T3) or Crinipellis sp. RCK-1 fermented diet (T4), whereas elevated levels of Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST) and Lactate dehydrogenase (LDH) were observed in the rats fed on the diet containing wheat straw fermented with P. cinnabarinus (T2). ALT is
commonly measured for evaluation of hepatocellular injury to determine liver health. When the liver is damaged or diseased, it releases ALT into the bloodstream, which makes ALT levels go up (Choletech Corp. Technical Bulletin, 2007). AST is an important enzyme in amino acid metabolism and found in the liver, heart, skeletal muscle, kidneys, brain and red blood cells and it is commonly measured clinically as a marker for liver health. Low levels of AST are normally found in the blood when body tissue or an organ such as the heart or liver is diseased or damaged, additional AST is released into the bloodstream. The amount of AST in the blood is directly related to the extent of the tissue damage (Choletech Corp. Technical Bulletin, 2007).

LDH is an oxidoreductase, which catalyzes the inter-conversion of lactate and pyruvate and most often measured to check for tissue damage e.g., hemolysis and others disorders i.e. cancer, meningitis, encephalitis, acute pancreatitis, and HIV (Coley et al., 1997). A typical range is 105–333 IU/L for LDH. The elevated level of stress enzymes and mycotoxins present in T2 diet may probably have contributed to the observed mortality and morbidity. Moreover, it is possible to induce a toxicological change due to simple alterations of dietary minerals or energy. These changes could be wrongly ascribed to biologically fermented feed; therefore precaution may be taken while formulation of the test diets as described by Villas-Boás et al. (2002).

Interestingly, feeding with diet fermented with Ganoderma sp. rckk02 (T3) and Crinipellis sp. RCK-1 (T4) has improved the nutrient utilization among rats and did not induce any stress on animals and hence appear to be safe for animal feeding.

**In vitro analysis for nutritional evaluation of animal feed**

Fungal fermentation of wheat straw brought about almost 30 and 10% improvement in in vitro dry matter digestibility, respectively, by Crinipellis sp. RCK-1 and Ganoderma sp. rckk02, which declined upon extending fermentation period. The findings of in vitro gas production test were consistent with our earlier observations, where ME and OMD in P. ostreatus and T. versicolor fermented straws were observed to be declined after 20th and 10th day, respectively, even after a continuous decrease in lignin content (Shrivastava et al., 2011). The decline in TDOM, OMD and ME after a certain
Discussion

period of SSF is normally attributed to the addition of degradable nitrogen compound to fiber rich feeds at the later stages of fermentation. Alternatively, it may have also resulted due to the improved capturing of nutrients, thereby causing higher production of microbial protein instead of gas and the diversion of carbon source for producing microbial protein than gas (Menke and Steingass, 1988; Shrivastava et al., 2011). Present findings are well in agreement with our earlier studies and other workers where, longer fermentation periods caused either no change or an undesired decrease in OMD, ME and Short chain fatty acids (SCFAs) (Shrivastava et al., 2011, 2012; Tuyen et al., 2012). Contrary to that, a consistent increase in digestibility with increase in fungal degradation of lignocellulosic components during prolonged incubations (30-60 days) has also been reported by several workers (Okano et al., 2006, 2009; Arora et al., 2011). Any decrease upon extending the fungal fermentation is reported to be primarily due to the extensive utilization of solubilized saccharides by the fungi, while lignin degradation might have already completed or could be due to the toxicity of extracts from the substrate for the rumen microorganisms (Zadrazil 1985; Capelari and Zadrazil, 1997). Thus the increase in in vitro substrate digestibility depends not only on the amount of lignin removed, but also on the availability of other sources of carbon in the substrate.

However, based on various studies it is widely accepted that the extent of lignin degradation and increase in in vitro digestibility largely depend on the fungus and incubation conditions and hence can not be generalized (Capelari and Zadrazil, 1997; Tripathi and Yadav, 1992; Tuyen et al., 2012). Recently Tuyen et al. (2012) have also shown that removing lignin alone does not always improve the gas production in syringe or in vitro digestibility and a weak correlation ($r = 0.47$) has been observed between them. Moreover, a strong and significant correlation have also reported between the increase in gas production and lignin to cellulose ratio (percent SSF efficiency in present study) and inclusion of hemicellulose loss in the analysis has shown to increase the goodness of fit of the equation (Tuyen et al., 2012).
Statistical optimization of SSF of wheat straw

Crinipellis sp. RCK-1 was chosen further to optimize the process of nutritive animal feed development owing to its property of fast growth and higher IVDMD improvement than Ganoderma sp. rckk02. Optimization of various factors becomes necessary in SSF processes since SSF is a polyfactorial event, in which the fungus, its enzymes, physical structure of substrate, physiological factors of fermentation and culture and nutritional conditions play an important role in controlling lignin degradation and digestibility of fermented substrate (Zadrazil and Brunnert, 1980). Nutrients limitation, deviation from optimum moisture, temperature, toxic metabolite generation of steric hindrance are crucial factors that decided the fate of SSF and may lead to change in product formation or undesirable spore generation (Moo Young et al., 1983; Oostra et al., 2001; Tengerdy and Szakacs, 2003; Mitchell et al., 2004). In the present study, the optimization of process majorly involved three steps; performing the statistically designed experiments, estimating the coefficients in a mathematical model and predicting the response and checking the adequacy of model.

First of all the effect of different carbon, nitrogen sources and phenolic compounds were studied using OFAT methodology. This study was taken up with the fact that production of ligninolytic enzymes majorly laccase is regulated by nutrients (carbon and nitrogen sources) and different inducers, which in turn affect transcription level of various lignin degrading enzymes (Pointing et al., 2005). Among different organic sources used, malt extract (5% w/w) was observed to be the most influencing in increasing the efficiency of SSF. While soybean meal and yeast extract least affected the efficiency of SSF. Addition of ammonium chloride (5% w/w) induced the fungus to attain maximum efficiency of SSF, while sodium nitrate had minimum effect on fungal growth among various inorganic nitrogen sources used. This may be attributed to the fact that complex nitrogen source release ammonium ions, which stimulates the microbial growth and enhance the enzyme, level (Slininger et al., 2009). Similarly, highest increase in lignin degradation and decrease in cellulose and hemicellulose after employing statistical designs has been observed by Sharma and Arora (2010b) under the optimized conditions (moisture 7ml/g, ammonium chloride 50 mg/g and
malt extract 50 mg/g). Similar increase in degradation of lignin and decrease in cellulose and hemicellulose degradation through statistical optimization has been reported by Arora and Sharma (2011). Use of certain nitrogenous supplements have already been known to enhance the ligninolytic enzyme production by white rot fungi and availing more organic matter for ruminant nutrition (Zadrazil and Brunnert, 1980; Mikiashvili et al., 2006; Sharma and Arora 2011).

Among different complex carbon sources/substrates, mixed fruit waste exhibited highest effect on efficiency of SSF, however it was a marginal increase and hence none of the carbon source/substrate was incorporated in further optimization process. Organic supplements contain sufficient reduced carbon and nitrogen and are rich in amino acids and simple sugars, which might be responsible for better enzyme production, fungal growth and substrate degradation (Reid, 1983). Similarly, none of the phenolic compound used i.e 2-6 DMP, Guaiacol, phenol, syringaldehyde, vanillin and 5-sulfosalicylic acid, showed any remarkable increase in efficiency of SSF by Crinipellis sp. RCK-1 and hence were not used in the future experiments. Similarly, aromatic compounds structurally related to lignin precursor have been reported to cause an increase in ligninolytic enzyme production by white rot fungi which eventually might help in increase in lignin degradation (Vasdev and Kuhad, 1994; Eggert et al., 1996; Xavier et al., 2007). In addition to them, xenobiotic response elements (XREs) are present in the region upstream from the laccasae promoter of fungi, which is regulated by aromatic compounds (Soden and Dobson, 2001; Xiao et al., 2004). But these inductive aromatic compounds are also often known to be toxic to fungal growth and metabolism as well, which could be due to the possible function of fungal laccase in polymerization of toxic aromatic compounds (Thurston, 1994).

Plackett - Burman methodology was then adopted to identify the most influencing physiological factors during SSF of wheat straw by Crinipellis sp. RCK-1. Among various factors screened under experimental design substrate to moisture ratio, incubation period and size of wheat straw were found to have maximal standard effect (ΣEi) on efficiency of SSF. The effect of these
factors can also be explained through having highest % contribution in deciding the efficiency of SSF. The effect of initial pH, humidity, critical depth, oxygen sparging and inoculum concentration was also studied but observed to be very nominal. The factors having lighter effect including temperature were kept at their optimum level as per the earlier experiments. However, the highest efficiency of SSF (30.57) obtained through PBD after 5 days of incubation, 1:2 (S:L ratio) and average straw size of 5 mm. Substrate to moisture ratio is a crucial factor in SSF in determining the growth of fungus as well as substrate degradation. The available water in SSF exist either in a complex form within the solid substrate or as a thin layer adsorbed to the surface of the particle or less tightly bound within the capillary region of the solid substrate (Cannel and Moo Young, 1980; Mudgett, 1986; Reid 1989; Manpreet et al., 2005). The moisture beyond optimum level inhibits the process through either causing a decrease in porosity due to the gummy texture of the substrate or altering the substrate particle structure leading to poor oxygen transfer and decreased diffusion (Mitchell et al., 2004). Contrary to that, lower moisture level beyond the optimum value leads to poor solubility of nutrients, inadequate swelling and higher water tension (Pandey, 2003; Krishna, 2005).

*Crinipellis* sp. RCK-1 grew optimally at 30 ºC, since the temperature has been known to have high effect on degradation of substrate organic matter and similarly majority of the white rot fungi have been found to degrade more organic matter at 25-30ºC (Zadrazil 1985; Capelari and Zadrazil, 1997; Tripathi and Yadav, 1992). Steric hindrance is an important limitation for fungal growth in SSF and the growing fungus must find accessible attack points on the substrate (Tengerdy and Szakacs, 2003). Straw size came out as very crucial factor in our study majorly due to the fact that substrate degradation during SSF largely depends on geometric position and proximity of substrate particles and the space required by branching mycelium (Laukevics et al., 1985). Moreover, the space utilization is directly linked with fungal growth and substrate packing density, which ultimately depends on straw particle size (Tengerdy and Szakacs, 2003).

Further, SSF of wheat straw by *Crinipellis* sp. RCK-1 was optimized using central composite design (CCD) of response surface methodology
Discussion

The response analysis revealed that highest efficiency of SSF was obtained when straw having average size of 7.5 mm, supplemented with 7.5% (w/w) of ammonium chloride and mixed with mineral salt solution at S:L ratio of 1:2.5 is fermented for 5 days at 30 ºC with 70 % relative humidity. RSM has the advantage over other statistical designs largely due to having understanding and modeling of both individual and interactive effects, which enables each reaction parameter to be optimized in coherence with others for achieving maximum response yield. Similar efforts have been made earlier to optimize SSF of wheat straw or agro residues using OFAT followed by other statistical designs (PBD and RSM) for production of animal feed. Tripathi and Yadav, (1992), applied OFAT method to study effect of culture conditions (type of inocula, initial pH, moisture content, periods), nutritional conditions (supplementation of urea, cattle urine, single super phosphate, molasses and whey) and substrate pretreatment (Physical, steaming, grinding; chemical, NaOH, Urea, urine). Basu et al. (2002) obtained highest desirability (0.705) on 6th day during SSF of wheat straw by *P. chrysosporium* using central composite design of RSM. In a very similar study, Bhatanagr et al. (2008), optimized SSF of wheat straw using CCD of RSM by *P. chrysosporium* in a 200 L staged vertical reactor. The fungus within 5 days, exhibited 30.90 % of lignin degradation under the optimized conditions (1.08 kg wheat straw; inoculum 0.38 g/100 g of wheat straw and 15 L/minute airflow). However, the lignin degradation was undesirably accompanied with fair amount of cellulose and hemicellulose degradation (24.99 and 27.19 %, respectively). Recently, attempts were made to enhance *in vitro* dry matter digestibility (IVDMD), lignin degradation and enzyme production by *Phlebia floridensis, Phlebia brevispora* during SSF of wheat straw and rice straw using Box-Behnken design of RSM (Sharma and Arora, 2010b; Arora and Sharma, 2011). The fungus could maximally achieve 29.6 % of lignin degradation at the expense of 14.9 % of hemicellulose and 11.6 % of cellulose, but the incubation period was too long i.e. 20 days (Sharma and Arora, 2010b). Moreover, in contrast to the study of Arora and Sharma (2011), where malt extract supplementation in conjunction with ammonium chloride significantly enhanced the maximum IVDMD and ligninolysis, no additional malt extract was supplemented in our study. However, the better
Discussion

selective ligninolysis capability of Crinipellis sp. RCK-1 could be because of the avoidance of the suppression of lignin degradation in the presence of excess nitrogen or switching of fungal metabolism towards lignin degradation instead of fungal growth in presence of nitrogen starvation (Commanday and Macy, 1985; Arora and Sharma, 2011). It is now widely accepted that effect of carbon and nitrogen source depends on the fungal strain and nature of compound /substrate tested (Mikiashvili et al., 2006; Arora and Sharma 2011). A detailed comparison about degradation of lignin, cellulose and hemicellulose by Crinipellis sp. RCK-1 and other fungi is given in Table 5.1. To the best of our knowledge, Crinipellis sp. RCK-1, under the present experimental condition was found to be most promising fungus for bioconversion of wheat straw in to highly nutritive and digestible animal feed.

Table 5.1. Maximum degradation of cell wall components in minimum time during solid state fermentation for animal feed development

<table>
<thead>
<tr>
<th>Fungus &amp; incu. period</th>
<th>% Lignin Degradation</th>
<th>% Cell. Degradation</th>
<th>% Hemi. Degradation</th>
<th>% CP improvement</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyathus stercorus (7)</td>
<td>-18.5</td>
<td>-2.5</td>
<td>-7.6</td>
<td>-</td>
<td>Agosin et al., 1985</td>
</tr>
<tr>
<td>Pycnoporus cinnabarinus (3)</td>
<td>-16.6</td>
<td>-3.6</td>
<td>-10.3</td>
<td>-</td>
<td>Agosin et al., 1985</td>
</tr>
<tr>
<td>Coriolus versicolor (7)</td>
<td>-17.3</td>
<td>-3.1</td>
<td>-</td>
<td>-</td>
<td>Zafar et al., 1989</td>
</tr>
<tr>
<td>Coprinus fimetarius (4)</td>
<td>-10</td>
<td>-27.5</td>
<td>-14</td>
<td>-</td>
<td>Kumar and Singh, 1990</td>
</tr>
<tr>
<td>Coprinus fimetarius (30+5) Karnal Process</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+ 280</td>
<td>Singh and Gupta, 1993</td>
</tr>
<tr>
<td>Trametes gibbosa(30)</td>
<td>-26.7</td>
<td>-7.32</td>
<td>-62.4</td>
<td>+41</td>
<td>Jalc et al. 1996</td>
</tr>
<tr>
<td>P. sajor caju (4)</td>
<td>-16.9</td>
<td>-23.4</td>
<td>-16.7</td>
<td>+64</td>
<td>Bisiain et al., 1997</td>
</tr>
<tr>
<td>Fusarium concolor (5)</td>
<td>-13.7</td>
<td>-7.62</td>
<td>-7.62</td>
<td>-</td>
<td>Li et al., 2008</td>
</tr>
<tr>
<td>P. chrysosporium (3) In reactor</td>
<td>-22.28</td>
<td>-23.93</td>
<td>-23.68</td>
<td>-</td>
<td>Bhatnagar et al., 2008</td>
</tr>
</tbody>
</table>
### Structural investigation of fermented feed

#### Structural characterization of fungal fermented feed by Electron Microscopy

Within 5 days of incubation *Crinipellis* sp. RCK-1 efficiently colonized and degraded wheat straw, as has been clearly demonstrated through surface micrograph of the unfermented and fermented wheat straw. The SEM micrograph of the edges of wheat straw revealed its degradation and distortion of tissues. Untreated wheat straw had a fibrous structure and clear demarcation between cells whereas, in fermented wheat straw middle lamella and S2 layer showed a clear separation of tissues as has been observed by Berrocal et al. (1997) during biological upgradation of wheat straw under SSF conditions by *Streptomyces cyaneus*. The degradation of

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>Untreated</th>
<th>Untreated - Fermented</th>
<th>Fermented</th>
<th>Fermented - Untreated</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. brevispora</em> (10)</td>
<td>-13</td>
<td>-2.5</td>
<td>-6.1</td>
<td>-</td>
<td>Arora and Sharma, 2009a</td>
</tr>
<tr>
<td><em>P. fascicularia</em> (10)</td>
<td>-13.1</td>
<td>-9.0</td>
<td>-4.1</td>
<td>-</td>
<td>Arora and Sharma, 2009a</td>
</tr>
<tr>
<td><em>P. floridensis</em> (10)</td>
<td>-12.6</td>
<td>-4.6</td>
<td>-6.7</td>
<td>-</td>
<td>Arora and Sharma, 2009a</td>
</tr>
<tr>
<td><em>P. radiate</em> (10)</td>
<td>-17.0</td>
<td>-3.8</td>
<td>-11.3</td>
<td>-</td>
<td>Arora and Sharma, 2009a</td>
</tr>
<tr>
<td><em>C. subvermispora</em> (10)</td>
<td>-15.7</td>
<td>-22.0</td>
<td>-12.7</td>
<td>-</td>
<td>Arora and Sharma, 2009b</td>
</tr>
<tr>
<td><em>P. floridensis</em> (20)</td>
<td>-29.6</td>
<td>-11.6</td>
<td>-14.9</td>
<td>-</td>
<td>Sharma and Arora 2010b</td>
</tr>
<tr>
<td><em>P. brevispora</em> (20)</td>
<td>-29.5</td>
<td>-15.9</td>
<td>-21.7</td>
<td>-</td>
<td>Arora and Sharma, 2011</td>
</tr>
<tr>
<td><em>P. ostreatus</em> (5)</td>
<td>-5.67</td>
<td>-2.41</td>
<td>-13.50</td>
<td>+8.9</td>
<td>Shrivastava et al., 2011</td>
</tr>
<tr>
<td><em>T. versicolor</em> (5)</td>
<td>-2.6</td>
<td>-7.97</td>
<td>-9.04</td>
<td>+14.0</td>
<td>Shrivastava et al., 2011</td>
</tr>
<tr>
<td><em>B. adusta</em> (49)</td>
<td>-42.6</td>
<td>-39.9</td>
<td>-44.3</td>
<td>+75.39</td>
<td>Tuyen et al., 2012</td>
</tr>
<tr>
<td><em>G. lucidum</em> (49)</td>
<td>-11.7</td>
<td>-4.4</td>
<td>-7.9</td>
<td>+16.75</td>
<td>Tuyen et al., 2012</td>
</tr>
<tr>
<td><em>L. edodes</em> (49)</td>
<td>-58.9</td>
<td>-9.4</td>
<td>-53.0</td>
<td>+38.74</td>
<td>Tuyen et al., 2012</td>
</tr>
<tr>
<td><em>P. eryngii</em> (49)</td>
<td>-31.8</td>
<td>-5.8</td>
<td>-27.5</td>
<td>+30.89</td>
<td>Tuyen et al., 2012</td>
</tr>
<tr>
<td><em>S. commune</em> (49)</td>
<td>-3.2</td>
<td>-15.6</td>
<td>-14.4</td>
<td>+29.84</td>
<td>Tuyen et al., 2012</td>
</tr>
<tr>
<td><em>V. volvacea</em> (49)</td>
<td>-0.1</td>
<td>-1.4</td>
<td>-7.5</td>
<td>+26.70</td>
<td>Tuyen et al., 2012</td>
</tr>
<tr>
<td>Ganoderma sp. rckk02 (5)</td>
<td>-11.78</td>
<td>-14.41</td>
<td>-11.51</td>
<td>+56.66</td>
<td>Shrivastava et al., 2012</td>
</tr>
<tr>
<td><em>Fomes fomentarius</em> (30)</td>
<td>-11.0</td>
<td>-</td>
<td>-</td>
<td>+22.3</td>
<td>Neifar et al., 2013</td>
</tr>
<tr>
<td><em>Crinipellis</em> sp. RCK-1 (5)</td>
<td>-25.84</td>
<td>-9.33</td>
<td>-9.75</td>
<td>+85.71</td>
<td>Present study</td>
</tr>
</tbody>
</table>
middle lamella and cell corners has supported the lignin degradation in wheat straw (Berrocal et al., 1997). The SEM image clearly exhibited the presence of interwoven mycelia of *Crinipellis* sp. RCK-1 on wheat straw surface as well as penetrated deep in straw. Similar observations of cell wall disruption in wheat straw and corn stover have been noticed by Kaparaju and Felby (2010), after characterization of wheat straw after oxidative and hydrothermal pretreatment.

The transmission electron (TEM) micrograph revealed the presence of fungal hyphae inside the cell lumen, which further confirmed the deep penetration of fungal hyphae and progressive degradation of wheat straw. Similar observations has been made during selective delignification of birch wood by *Cerrana unicolor*, *Ganoderma applanatum*, *Ischnoderma resinosum*, *Poria medulla-panis* (Blanchette et al., 1985). However, unlike various other studies, *Crinipellis* sp. RCK-1 has shown potential to selectively degrade lignin irrespective of the tissue ranging from most lignified xylem and sclerenchyma to less lignified parenchyma as has been reported by Akin et al. (1995) and Berrocal et al. (1997). It was further elaborated that major delignification was carried out in middle lamella, which caused a defibracation of cells and progressive thinning of all cell wall layers towards middle lamella caused erosion trough or holes to form. However, in contrast to the selective degradation phenomenon, other secondary layers i.e. S1 and S2 are also majorly degraded in simultaneous type of fungal decay (Blanchette et al., 1985; Karunananda et al., 1995; Lequart et al., 2000). These observation clearly demonstrated the robust nature of *Crinipellis* sp. RCK-1 and its potential to convert poor quality wheat straw in to nutritive animal feed without affecting much of cellulose and hemicellulose portion of cell wall. The electron dense residuals of lignin termed here as lignin aggregates have also been demonstrated earlier by many workers and it is considered to be a common characteristic of advanced stage of white rot decay (Berrocal et al., 1997; Barrasa et al., 1995; Lequart et al., 2000).

**Fourier transform infrared spectroscopy (FTIR) Analysis**

The infrared spectrum of unfermented and *Crinipellis* sp. RCK-1 fermented wheat straw revealed a slight peak disintegration at 899 cm$^{-1}$, which denotes
degradation in cellulose content. In addition to that representative peaks, which denotes lignin breakdown/modification and aromatic ring stretch have been noticed in FTIR spectra of fermented wheat straw. Moreover, peak disintegration (1375 cm\(^{-1}\)) was noticed, which exhibit C-H bond deformation in cellulose and hemicellulose. Peak smoothning at 1158 cm\(^{-1}\) was also found which denotes C-O-C vibration (found in cellulose and hemicellulose). The region from 1800 – 4000 cm\(^{-1}\) did not show any useful information except broad hydroxyl and aliphatic C-H absorption as has been described by Buta et al. (1989). The microbial degradation of lignin has been found to involve oxidative depolymerization, prefential removal of syringyl groups and an increase in carbonyl and carboxylate groups (Crestini et al., 1998; Vane et al., 2001). As observed in our study it has been proposed that wheat straw may also undergo aromatic ring cleavage during the early stages of biodegradation (Crestini et al., 2001) Similar FTIR spectra has been observed by Buta et al. (1989) while studying lignin degradation in wheat straw by Stropharia rugosoannulata and compared with reference lignin. While peak observed in spectra from Crinipellis sp. RCK- at 1040 cm\(^{-1}\) and 1640 cm\(^{-1}\) are well in accordance of study of Dorado et al. (1999), exhibiting selective lignin degradation of wheat straw by C. subvermispora.

**Thermogravimetric analysis**

Thermal analysis is convenient, reproducible and a very useful method for characterizing heterogenous organic material. In particular, it is a valuable analytical technique to investigate the physicochemical properties of macromolecules such cellulose. The resistance of biomass to enzymatic attack is majorly due to the highly resistant crystalline nature of cellulose, lignin encrustation and site available for enzymatic action (Fan et al., 981). A decrease in thermal decomposition temperature occurred due to the fugal treatment of wheat straw, which is in favor of thermal degradation. Yang et al. (2010) noted similar decrease in thermal decomposition temperature, with increase in heating rate during characterization of enzyme acid hydrolysis lignin of wheat straw.
Powder X ray diffraction analysis

Powder X ray diffraction analysis of unfermented and fermented wheat straw revealed that *Crinipellis* sp. RCK-1 has significantly altered/ modified the wheat straw structure, especially in terms of cellulose. The decrease in degree of crystallinity of cellulose in wheat straw could be due to fungal fermentation, which eventually would have helped in improving digestibility of fermented feed in rumen. Similar findings have been reported by Saha et al. (2010), when crystallinity of jute fibers was reduced through alkali stem treatment. Crystallinity index is generally measured to assess the ordered orientation of cellulose crystallites and it could increase due to removal of amorphous cellulose (Kumar et al., 2009). However, the decrease observed in crystallinity index during SSF by *Crinipellis* sp. RCK-1 could be attributed to the intact cellulose or a slight decrease in crystalline cellulose instead of amorphous cellulose, which eventually helped in preserving cellulose and developing nutrition rich animal feed. Smaller crystallinity denotes the poor orientation of crystallites of cellulose and is possible due to damage to cell wall (Ansell and Mwaikambo, 2002). Similar findings have been reported by Yang et al. (2009), where anaerobic digestion destroyed the crystalline structure of *Spartina alterniflora* and brought down crystallinity index from 0.510 (undigested) to 0.479.

Scale up of the animal feed development

Scale up of the animal feed development was carried out to test the possibility of scalability and also to produce larger quantity of fermented feed for conducting large scale *in vivo* animal feeding trial. An increase in substrate degradation was observed when SSF was scaled up from 100 g to 25 Kg in Koji room. Lignin degradation was found to be improved from 25 % in at 100 g level to 26.43, 33.84, 34.25% at 500 g, 5 Kg and 25 Kg levels, respectively. There was an increase observed in lignin degradation after increasing the experiment scale however, along with lignin it also caused an undesirable increase in cellulose and hemicellulose degradation. The comparatively higher decrease in cellulose and hemicellulose lead to a substantial decrease in efficiency of SSF. The decrease observed in efficiency of SSF especially at 5 kg and 25 kg level could be attributed to an overall
decrease in dry matter loss, which might have caused a relatively higher decrease in cellulose and hemicellulose. Similarly, when SSF of wheat straw was attempted at levels of 150 g in 7 L and 60 kg in 1200 L solid state bioreactor, a drastic decrease in lignin degradation (6-9%) was observed. Moreover, the degradation of cellulose and hemicellulose in SSF bioreactor remained almost similar to the SSF experiments carried out in Koji room except a drastic decrease observed in hemicellulose in 7L bioreactor. Consequently, the efficiency of SSF in case of SSF bioreactors went down severely, which could also be attributed to the least dry matter loss of substrate obtained in bioreactor. Literature on SSF of substrates in a bioreactor pertaining to animal feed development is scanty and limited efforts have been made earlier (Grant et al., 1978; Kumar and Gomes, 2008; Bhatanagar et al., 2008). Grant et al. (1978) carried out a semisolid fermentation of 330 kg ryegrass straw in a pilot plant (2,270 L capacity) using *Candida utilis* for 12-96 hour. In their study, the degradation of lignin followed same trend like the present study and was found depressed with considerably higher degradation of hemicellulose. They have also observed an increase in crude protein content but the whole degradation and efficiency of the process was inferior compared to laboratory scale fermentation. Moreover, the depressed fungal degradation in our study could also be a result of variable oxygen concentration at different location in bioreactor as pointed out by Grant et al. (1978). Likewise, *P. chrysosporium* has also been reported to degrade 27% of lignin within 5 days of incubation in a vertical staged bioreactor but a simultaneously a degradation of 29% in cellulose was also observed (Kumar and Gomes, 2008). Till date, among several designs, Plafractor bioreactor designed by Biocon Ltd has been partially successful and It was hypothesized thereby, that no single reactor design can solve all the problems in solid state bioconversion processes (Suryanarayan and Mazumdar, 2001; Kumar and Gomes, 2008).

As the fungus metabolizes, the O₂ consumption gradually increase with simultaneous liberation of CO₂. This is generally accompanied by an increase in temperature due to exothermic reactions. This stage can be assumed as exponential phase of fungal growth and could explain highest lignin degradation (Agosin et al., 1985). The CO₂ evolution pattern observed
Discussion

in the study of Grant et al. (1978) followed similar pattern to the present report and it prevailed between 12-29 hour after inoculation and declined thereafter and coincided with maximum the fungal growth. The heat and mass transfer within the solid matrix is reported to be one of the major problems in solid state bioconversion studies due to poor conductivity of lignocellulosic residues (Ashley et al. 1999; Kumar and Gomes, 2008). Temperature check in our study revealed that the temperature in different layers of substrate was marginally different, which could be either due to the better heat and mass transfer in the bioreactor in present study or comparatively poor growth of *Crinipellis* sp. RCK-1. Since, temperature has been found to increase from 29 ºC to 45 ºC in pilot plant SSF of ryegrass straw earlier (Grant et al. 1978).

Consistent efforts were made by several workers to produce large quantity of fermented feed under SSF conditions but most of them could not reach up to commercial level majorly due to either slow growth of fungi vis a vis a longer periods of fungal fermentation or lower efficiency of degradation. Among these reports, SSF composting of substrate capacity of 1000 tonnes/week of fully grown compost) using *A. bisporus* (Zadrazil et al., 1985); SSF of wheat straw at 50 kg level by *Coprinus fimetarius* and *Pleurotus ostreatus* (Singh and Gupta, 1986 (Karnal Process); Kumar and Singh, 1990; Tripathi and Yadav, 1992), 100 Kg level by *P. ostreatus*, *P. sajor caju* and *Pleurotus of Iranian tissue* (Rouzbehan et al., 2001) and 500 g level by *Ganoderma* sp. rckk02 (Shrivastava et al., 2012), are the major efforts. However, SSF by *Crinipellis* sp. RCK-1 at 500 g level in trays in Koji room, were finally chosen for large scale animal feed development majorly due to its ease of handling, higher lignin degradation (26.43%), efficiency of SSF (37.35) and increase in crude protein (90.23%). The improvement in substrate colonization and crude protein during scale up studies of SSF of wheat straw could be attributed to the improved aeration and maintenance of humidity in Koji room, which might have also facilitated higher substrate degradation.
**In vivo evaluation of fermented feed**

Nutritional upgradation of *Crinipellis* sp. RCK-1 5 day fermented feed was also assessed through estimation of fungal biomass (ergosterol), total crude protein and amino acids content. However, estimation of fungal biomass is a major problem while determining fungal growth especially in solid state fermented products, it is because the biomass grows and remain trapped inside the substrate. Fungal biomass enrichment was analyzed by total ergosterol estimation of unfermented and *Crinipellis* sp. RCK-1 5 day fermented wheat straw. *Crinipellis* sp. RCK-1 enhanced fungal biomass manifold in fermented feed i.e. up to 5370 µg/g of dry substrate from 32 µg/g in the unfermented wheat straw. The evaluation of ergosterol content as fungal biomass index holds specific importance due to its sensitivity, majorly because of the presence of rare 5-7 double bonding based assay method. Since this bonding is rarely reported in major sterols of plants (Newell et al., 1988). Ergosterol content has been established a reliable indicator of fungal growth under SSF conditions and it majorly represent the live biomass (Gesnner et al., 1991; Han et al. 2005; Brozzoli et al., 2010). In a similar study during nutritional upgradation of cornmeal by *Ganoderma lucidum*, ergosterol yield of 6.9µg/g dry substrate on 25th day indicated the fungal biomass enrichment in the fermented feed (Han et al., 2005). In a very recent report pertaining to animal feed development Brozzoli et al. (2010) have reported an increase in fungal biomass (ergosterol) after fermentation of stoned olive pomace; a very unconventional feedstuff. The olive pomace was mixed with wheat bran, wheat middlings, barley grains, crimson clover, wheat flour shorts and field beans and fermented with *Pleurotus* sp. for 6 weeks and an ergosterol yield of 21-23 µ/g ds was reported (Brozzoli et al. 2010). However, in both of these studies a significantly lesser yield of ergosterol was observed compared to *Crinipellis* sp. RCK-1. This could be attributed to the kind of inoculum used i.e. Han et al. (2005) have used mycelium discs and Brozzoli et al. (2010) used immobilized inocula, and eventually the growth might have been comparatively less. In present study, enrichment of fermented feed with enormous fungal biomass displays development of an efficient bioprocess of wheat straw bioconversion, providing favorable
environment for a luxuriant growth of fungus within substrate, eventually producing more digestible and nutrient rich cattle feed.

Amino acid profile of fermented wheat straw revealed that fungal fermentation caused a significant increase in total amino acid content as well as the content of certain essential amino acids. Essential and non-essential amino acids are generally discriminated on the basis of metabolic capability of organisms to synthesize it or not. Rumen microbes are capable of synthesizing amino acids for microbial protein synthesis only when sufficient carbon source (Majorly from dietary carbohydrate), non-protein nitrogen (from inorganic supplementation) and inorganic sulfur are available (Atasoglu et al., 1998). It is generally established that ruminants do not have a dietary requirement of amino acids because the rumen microbes synthesize them sufficiently. However, *in vitro* studies have indicated that for many rumen microbes to grow and function at maximum rate, intact amino acids and peptides supplied by diets are obligatory requirement (Cotta and Russell, 1982; Atasoglu, 1999, 2003). Similar increase in individual and total amino acid contents through fungal fermentation have been reported by various workers and rather it has been incorporated as an essential part of fermented diet characterization (Penaloza et al., 1985; Valmaseda et al., 1991; Singh and Gupta, 1993; Peigi et al., 1997; Han et al., 2003) Thus fermentation of wheat straw by *Crinipellis* sp. RCK-1 has added sufficient nutrients to support microbial protein synthesis, eventually causing an increase in essential amino acids in the feed.

*In vitro* analysis of *Crinipellis* sp. RCK-1 fermented feed based diets depicted that even after reduction of 50 % grains from concentrate mixture in diet, which in turn reduced 0.34 Mcal/kg dry matter from concentrate mixture, no significant decrease was observed in OM digestibility, metabolizable energy and microbial biomass production. This clearly indicated that *Crinipellis* sp. RCK-1 5 day fermented feed has potential to replace 50 % grains from traditional feed concentrates and paved the way for *in vivo* evaluation of feed. *In vitro* gas production (IVGP) has been reported to be advantageous over *in vivo* methods, being less expensive, less time consuming, requiring small amount of sample, better quantification of nutrient utilization and accuracy in describing digestibility (Menke and
Steingass, 1988; Sallam 2005). But to ensure the suitability of fermented feed, in vivo digestion trials are essential and hence it was attempted to establish its application in evaluation of cattle feed (Menke and Steingass, 1988; Sallam, 2005; Shrivastava et al., 2012;).

Similar to the present study, an increase in DMI (% of body weight/day) has been observed in lambs when fed with P. sajor caju fermented wheat straw (Calazada et al., 1987). In a similar feeding trial using Murrah male buffalo, Kakkar and Dhanda (1998) replaced wheat straw or rice straw (control diet) with Pleurotus treated spent wheat or rice straw at a rate of 0% (T1), 50% (T2) and 100% (T3). They observed an increase in DMI in case of T2 diet fed calves compared with T1 diet fed group. Likewise, similar trend for dry matter intake have also been noticed by Fazaeli et al. (2006) in parameters such as DMI (g/d), OMI (g/d), DMI (g/kg BW^{0.75}), OMI (g/kg BW^{0.75}), dDMI & dOMI (g/d) and dDMI & dOMI (g/kg BW^{0.75}), when male sheep were fed with fermented wheat straw treated with different Pleurotus strains.

The highest values for nutrients digestibility were observed in T-2 (Crinipellis sp. RCK-1 fermented feed based diet), which was significantly higher than other treatments. Similar results have been observed by many other workers while feeding fungal fermented diets to the monogastric animals and ruminants i.e. lambs, goats, buffalo calves, Simmenthal heifers, Sheep and Hanwoo steers (Okano et al., 2009, Kakkar and Dhanda, 1998; Adamovic et al., 1998; Calzada et al., 1987; Fazaeli et al., 2006; Gupta et al., 1988a; Oh et al., 2010). However, non-significant improvement in in vivo digestibility for certain nutrients i.e. crude protein and ether extract has also been reported by Okano et al. (2009) and Shrivastava et al. (2012).

In contrast to our findings a decrease in TDN was observed when chemically treated (UTRS) and fungal treated (FTRS) rice straw were fed to cross bred goats i.e. 51.28 and 38.38%, respectively (Gupta et al., 1988a). This decrease was linked with a higher dry matter loss and loss of potential energy materials by Coprinus fimetarius during prolonged fungal fermentations. According to this study, the increase in TDN content clearly demonstrates the improved and intact availability of energy rich
carbohydrate components in *Crinipellis* sp. RCK-1 fermented Feed. The gradual increase in DCP in T2 and T3 dietary groups represented an improved assimilation of nitrogen by ruminants as has been reported by Kakkar and Dhanda (1998). Interestingly, in present study T3 group, where 50% grains from concentrate mixture have been replaced by *Crinipellis* sp. RCK-1 5th day fermented feed, fed to calves, exhibited maximum DCP (8.8%) and also improved TDN, which clearly demonstrated the possibility of using T3 diet as a sole ration to the ruminants. The results of present feeding trial are consistent with our earlier report, where TDN and DCP were found to be increased upon feeding *Ganoderma* sp. rckk02 fermented wheat straw based diet to crossbred goats (Shrivastava et al., 2012).

Growth rate in terms of average daily gain (ADG) of calves was also found effectively improved upon feeding *Crinipellis* sp. RCK-1 fermented Feed. In a similar study Dey et al. (2004) have also reported improvement in ADG, when an anaerobic fungus, *Orpinomyces* sp. was administered at the rate of 106 CFU/ml/calf/week (Diet T2) in addition to wheat straw + concentrate feed based diet (T1). The improved ADG among calves fed on Diet T2 (709 g/d) compared to diet T1 (614 g/d) was attributed to the better utilization of fermented wheat straw because of the availability of more digestible carbohydrates (Shrivastava et al., 2012, Dey et al., 2004). Likewise Salman et al. (2008) have also shown that feeding of fermented sugar beet pulp supplemented diet @ 0.6% (T3) and 0.9% (T4) to goats, significantly improved ADG up to 95.25 and 104.83 g/h/d, respectively, compared to control diet (T1, unsupplemented) i.e. 88.58 g/h/d. However, their study also clearly revealed that at the lower rate of supplementation i.e. 0.3% (T2), ADG could be improved but non – significantly, as in case of our study. *In vivo* feeding trials pertaining to grain replacement have not been found much successful in past and a decrease in ADG has also been reported, which could largely be due to the problem of palatability and reduced feed conversion (Adamovic et al., 1998). Adamovic et al. (1998) observed a significant decrease in ADG when 44% grains replaced diet fed to the calves, while we could observe a marginal decrease in ADG of calves fed with T3 diet (50 % grain replaced). Feed conversion ratio in case of T3 diet was maximum compared to T2 and T1 diets, which demonstrates the improvement in
average daily gain, feed intake and feed conversion as has been reported by Salman et al. (2008). Decreased feed conversion is undesirable and are known to found associated with depressed average daily gain by ruminants (Adamovic et al., 1998). The better assimilation of nutrients and adequate supply of amino acids supported not only the maintenance but also resulted in an improved body weight gain among animals fed on fermented diets.

Similar to our findings elevated levels of blood urea nitrogen (BUN) utilization, total protein, serum creatinine and albumin have indicated an improved protein utilization in calves in T2 and T3 diet groups (Oh et al., 2010). These observations were found within range and did not have any adverse effect on health of calves. Present study showed that no major and significant differences were noticed in blood metabolites among different dietary groups. It is apparent that *Crinipellis* sp. RCK-1 5 day fermented feed based diets can replace the conventional diets and grains in it without causing any adverse effect.

Our study has clearly demonstrated that the fungus *Crinipellis* sp. RCK-1 has tremendous potential in degrading lignin and not affecting much of cellulose and could successfully improve the nutritional quality of crop residues like wheat straw.