2. Review of Literature

2.1. Lignocellulosics

Lignocellulose is the major component of biomass, comprising around half of the plant matter produced by photosynthesis and representing the most abundant renewable organic resource. It consists of three types of polymers, cellulose, hemicellulose and lignin that are strongly intermeshed and chemically bonded by non-covalent forces and by covalent cross linkages (Pérez et al., 2002). Lignocellulose is generated by forest herbs, shrubs, grasses, plants and trees, while a variety of crop residues including legumes, cereals, fruits and vegetables are generated as agricultural residues. Forest residues are degraded by a variety of organisms present naturally in the ecosystem and the complex biomass is recycled in the nature with time (Arantes et al., 2010). On the other hand, the residues generated as agricultural waste or as a by product of crop, fruits and vegetable processing industries are difficult to manage as the amount is very large depending upon the need of human society which is increasing day by day. Only a small amount of the cellulose, hemicellulose and lignin of these plant products is used as raw material for different purposes including animal feed and rest being considered waste (Sánchez, 2009).

2.1.1. Basic composition of lignocellulosics

Cellulose, hemicellulose, and lignin are the main constituents of lignocellulosic materials. Apart from these primary polymers, plants comprise other structural polymers e.g. waxes, proteins etc. (Malherbe and Cloete, 2002). Major part of lignocellulosics is mainly contributed by the plant cell wall. Plant cell wall is broadly divided into primary and secondary cell walls. Between the cells, there is a component that acts as glue to join the cells together, it is known as middle lamella. The primary wall can be divided into an outer and an inner surface. Following the primary wall, the secondary wall is present, which consists of three layers: outer layer (S1), middle layer (S2) and inner layer (S3) (Figure 2.1). The secondary walls usually account for more than 95% by weight of the cell wall material. It is composed of cellulose (40-50%), hemicellulose (20-30%) and lignin (20-30%), and therefore referred to as lignocellulosic feedstock (Fengel and Wegener 1989).
Distribution of lignocellulosic biomass varied, depending upon the plants as well as different parts of plants. Tropical forages tend to have higher cell wall content than temperate forages, and as a consequence, higher cellulose and hemicellulose contents.

Approximately equal amounts of pectin and hemicellulose are present in legumes primary walls whereas hemicellulose is more abundant in grasses. The secondary walls of woody tissue and grasses are composed predominantly of cellulose, lignin, and hemicellulose. The cellulose fibrils are embedded in a network of hemicellulose and lignin. The secondary walls of xylem fibers are further strengthened by the incorporation of lignin. Beside these complex polymers of cell wall, other simple components are also present which consists of proteins and simple sugars and easily digestible water soluble fraction of lignocellulose.
2.1.2. Water soluble part

Water soluble part of plant cell wall mainly comprises of some proteins and polysaccharides soluble in cold or hot water. These soluble polysaccharides are associated with cellulose microfibrils. This network formation implies the setting of interactions between the wall polysaccharides. It contains pectic substances, found in the primary cell wall and middle lamella of the plant tissues as complex polymers of galacturonic acid, which play an important nutritional role in the gastrointestinal tract. Their dietary function is due to their physical properties, which include the ability to form gels, to build cations and to increase the water holding capacity (Voragen et al., 2001; Bailoni et al., 2003).

2.1.3 Cellulose

The main polysaccharide in plant biomass is cellulose (Figure 2.2). Cellulose is a homopolysaccharide composed of D-glucopyranose units linked to each other by β-(1→4) glycosidic bonds (Figure 2.3) (Sjöström, 1993; Laine, 2005).

The molecules are completely linear and have a strong tendency to form intra and intermolecular hydrogen bonds (Bochek, 2003). This leads to bundling of cellulose molecules into microfibrils, which in turn form fibrils and finally cellulose fibers. Cellulose is usually arranged in microcrystalline structures, which is difficult to dissolve or hydrolyse under natural conditions (Rismani-Yazdi, 2008).

Figure 2.2 Lignocellulose composition (Ritter, 2008)
2.1.4. Hemicellulose

The non cellulose portion of cell wall carbohydrate is a complex substrate containing a variety of sugars and linkages. Hemicellulose is more soluble than cellulose. Xylan is the most common hemicellulose component of grass and wood. Xylose is always the sugar monomer present in the largest amount, but mannanuronic acid and galacturonic acid also tend to be present. Hemicellulose is a heteropolysaccharide and contains many different sugar monomers along with xylose it includes mannose, galactose, rhamnose and arabinose.

Xylan has a backbone of $\beta$-(1→4) linked xylopyranose units (Figure 2.4) (Sjöström, 1993). Single-unit side chains are 4-O-methyl-D-glucuronic acid units attached by $\alpha$-(1→2) bonds, on average one unit per 5-6 xylose units, and L-arabinose units attached by $\alpha$-(1→3) bonds, on average one unit per 5-12 xylose units (Laine, 2005).

![Figure 2.4 Structure of major softwood hemicellulose (Xylan)](image)
2.1.5. Lignin

Lignin is a highly irregular and insoluble polymer consisting of phenylpropanoid subunits, namely p-hydroxyphenyl (H-type), guaiacyl (G-type), and syringyl (S-type) units (Figure 2.5).

![Figure 2.5 Precursors of lignin polymers](image)

It is synthesized by one-electron oxidation of the precursors; p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, generating phenoxy radicals which then undergo nonenzymatic polymerization. These unspecific reactions create a high-molecular-weight, heterogeneous, three-dimensional polymer. Lignin polymer comprises of a variety of monomers connected by various C–C and C–O–C nonhydrolyzable bonds with irregular arrangement of successive monomeric and intermonomeric bonds (Figure 2.6). Ether bonds between propyl side chains and aromatic nuclei (arylglycerol-β-aryl or β-O-4 ether) constitute the major parts. Carbon–carbon bonds which occur primarily between aromatic nuclei and propyl side chains (diaryl propane or β-1 bond) are less frequent (Alder, 1977).

Unlike cellulose or hemicellulose, no chains containing repeating subunits are present, thereby making the enzymatic hydrolysis of this polymer extremely difficult.
Figure 2.6 Structure of a lignin polymer

The composition of plant cell wall fibers varies, depending upon the type of lignocellulose (Table 2.1)

Table 2.1 Typical composition of various lignocellulosic materials (Betts et al., 1991)

<table>
<thead>
<tr>
<th>Lignocellulosic material</th>
<th>Lignin</th>
<th>Cellulose</th>
<th>Hemicellulose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hardwood</td>
<td>18-25</td>
<td>45-55</td>
<td>24-40</td>
</tr>
<tr>
<td>Softwood</td>
<td>25-35</td>
<td>45-50</td>
<td>25-35</td>
</tr>
<tr>
<td>Grasses</td>
<td>10-30</td>
<td>25-40</td>
<td>25-50</td>
</tr>
</tbody>
</table>
2.1.6. Ash and Minerals

Calcium (Ca), phosphorus (P), magnesium (Mg), and potassium (K) values are expressed as a percentage of each in the feed. One of the major mineral components of straws is silica, particularly in rice and in the leaves fraction. It must be emphasized the negative effect of high concentrations of silica, as in rice straw, inversely correlated with polysaccharides degradability in the rumen (Agbagla-Dohnani et al., 2003).

2.2. Animal Feed

Animal feed is the foodstuff, which is used to feed livestock such as cattle, cows, buffalos, goats, sheep, horses, chickens and pigs. Fodder refers particularly to plant residues given to the animals. It includes hay, straw, silage, compressed and pelleted feeds, oils and mixed rations, and also sprouted grains and legumes. Forage is plant material (mainly plant leaves and stems) eaten by grazing livestock (Ali et al., 2011).

2.2.1. Use of Agricultural residues as feed

Forage crops occupy approximately 5% of the total agricultural fields, which is not sufficient enough to fulfil today’s requirements. Agricultural residues include crop residues remaining in fields after harvest (primary residues) and processing residues generated from the harvested portions of crops during food, feed, and fiber production (secondary residues).

A variety of agricultural residues including sugarbeet residues, lentil crop residue, faba bean leaf and stems, cotton stalk, corn stover, sugar cane bagasse, wheat straw, paddy straw and barley straw are generally used as feed and feed supplement. Most of these residues have their own limitations such as sugarbeet residues are assumed to amount only 0.1 kg for each kilogram of raw beet harvested, which is quite a lesser amount to fulfill the requirements. Barley is generally grown in drier areas where feed is in shorter supply relative to livestock numbers. Thus, the use of these residues is limited because of their availability in particular region while cereal crops generate huge residues, which are lower in their nutritive quality as feed (Nordblom, 1988; Wirsenius, 2003).
Wheat (*Triticum aestivum*) and paddy (*Oryza sativa*) are important cereal crops. Agricultural fields of north western zone (NWZ), north eastern zone (NEZ), and the central zone (CZ) of India are the main contributors of wheat production, while humid subtropical and tropical wet & dry land including northern, eastern and southern zones of India are main paddy producer regions (Gill *et al.*, 2008).

The fibrous by-products of these cereal straws are very abundant and likely to increase in quantity in the future because of the surging need to produce more and more cereal grains for human consumption. Straws are almost entirely made of cell-walls, which comprises highly lignified structural carbohydrates and of small amounts of structural proteins and minerals (Antongiovanni and Sargentini, 1991). In straws, cellulose and xylan hemicellulose are the predominant components. Few pectic compounds and few mannans are also present.

### 2.2.2. Fiber content

In nutrition, the term fiber refers to the components of plant derived foods and feedstuffs that are not digestible by mammalian enzyme systems (Moore and Hatfield, 1994). In forages commonly fed to livestock, fiber refers to the plant cell wall. Mammals do not possess the enzymes to hydrolyze the predominant β1-4 linked polysaccharides that occur in cell walls and depend on microorganisms in the gastrointestinal tract to ferment these polysaccharides to absorbable nutrients (Jung, 1997).

Quality of feed depends upon its composition e.g. neutral detergent fiber (NDF), acid detergent fiber (ADF), water solubles, hemicellulose, cellulose, lignin, protein and ash content, where higher ADF value and lignin content results in less digestibility (Garcia *et al.*, 2003). The total fiber content of forage is contained in the neutral detergent fibres (NDF) or cell walls. This fraction contains cellulose, hemicellulose, and lignin, while acid detergent fibers (ADF) consist primarily of cellulose, lignin, and acid detergent fiber crude protein.
2.2.3. Role of chemical constituents in digestibility of plant cell wall

Composition and constituent of cell wall plays an important role to determine the digestibility. Richness in holocellulosic components enhances the digestibility. However, lignin plays a major role in protecting the substrate from microbial and chemical attacks as this is the most resistant part in plant cell wall.

The high level of lignification and silicification limits ruminal degradation of the carbohydrates and the low content of nitrogen are the main deficiencies of rice straw, affecting its value as feed for ruminants (Van Soest, 2006).

Ash constitutes the inorganic matter mainly containing minerals which is also difficult for animal digestion but required in trace amounts. As ash does not contain any energy, so it would naturally lower the overall energy and digestibility of the feed (Fonnesbeck et al., 1981). Minerals also act as barrier to the attack of rumen microbes to structural carbohydrates.

The physical barrier concept is best described by making use of the rumen as the rumen being an anaerobic environment, it is clear that access and attachment of the microorganism to the substrate is vital if efficient cellulose hydrolysis is to be effected (Malherbe and Cloete, 2002).

2.3. Lignin as a barrier

Lignin degradation is very slow under anaerobic conditions and depending on pressure and time scale, leads to the accumulation of humus to form peat, organic soil matter, lignite, and coal (Heider and Fuchs, 1996). Polymeric lignin remains stable in anaerobic environments like rumen for long periods of time (Egland et al., 1997; Malherbe and Cloete, 2002).

Lignin is a complex substance covalently bound to side chains of xylans of cell-walls. It represents an obstacle to microbial digestion of structural carbohydrates, both because it is a physical barrier and because of the depressing effect on microbial activity, due to the phenolic compounds it contains (Antongiovanni and Sargentini, 1991). Thus, along with its indigestibility, lignin binds hemicellulose and cellulose to form a matrix and make these energy rich components inaccessible for the ruminants.
Following lignin-carbohydrate bonds have been well defined (Watanabe, 2003; Laine, 2005):

- A benzyl ether type between the α-hydroxyl group of a lignin unit and a hydroxyl group of a carbohydrate
- A benzyl ester type between the α-hydroxyl group of a lignin unit and a carboxylic acid group of a carbohydrate
- A glycoside type between an aliphatic or aromatic hydroxyl group and the reducing end group of carbohydrates
- An acetal type between two hydroxyl groups of carbohydrates and a carbonyl group of lignin

![Benzyl ether type](image1)

![Glycosidic type](image2)

![Benzyl ester type](image3)

![Acetal type](image4)

**Figure 2.7** Proposed structure of the different bonds between lignin and hemicelluloses (Watanabe, 2003).
In wheat straw cell walls, the majority of lignin is directly linked to arabinose side chains of xylan by ether bonds without hydroxycinnamic acids. A particular aspect of wheat straw cell walls is the existence of a non-core lignin which represents up to 20% of total lignin. The removal of this particular lignin generally increases the digestibility of the material and enhances further microbial or enzymatic bioconversion (Durot et al., 2003).

2.4. Factors governing the chemical constituents of straw

Plant constituents may also vary with respect to the climate, season, soil quality, fertilizers and temperature etc., which are responsible for the difference in their quality and digestibility (Ford et al., 1979; Ouédraogo-Koné et al., 2008; Whalley et al., 2008). The composition and concentration of these fibers may vary according to their place of origin and climatological conditions (Table 2.2).

**Table 2.2** Chemical composition of wheat and paddy straw (%) of different geographic locations

<table>
<thead>
<tr>
<th>Region</th>
<th>Lignin</th>
<th>Cellulose</th>
<th>HMCL</th>
<th>Ash</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat straw</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>China</td>
<td>5.3</td>
<td>35.1</td>
<td>27.1</td>
<td>6.04</td>
<td>Hongzhang and Liying, (2007)</td>
</tr>
<tr>
<td>Greece</td>
<td>16.4</td>
<td>32.1</td>
<td>29.2</td>
<td>4.8</td>
<td>Papatheofanous et al., (1998)</td>
</tr>
<tr>
<td>India</td>
<td>24</td>
<td>32.5</td>
<td>35.3</td>
<td>7</td>
<td>Arora and Sharma, (2009)</td>
</tr>
<tr>
<td>USA</td>
<td>8.2</td>
<td>48.6</td>
<td>27.7</td>
<td>6.7</td>
<td>Saha et al., (2005)</td>
</tr>
<tr>
<td>Paddy straw</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>China</td>
<td>19</td>
<td>44</td>
<td>20.1</td>
<td>9.8</td>
<td>Deng et al., (2007)</td>
</tr>
<tr>
<td>China</td>
<td>8.6</td>
<td>30.4</td>
<td>32.3</td>
<td>6.3</td>
<td>Jin and Chen, (2006)</td>
</tr>
<tr>
<td>India</td>
<td>20.3</td>
<td>40</td>
<td>29.2</td>
<td>10.2</td>
<td>Sharma and Arora, (2010)</td>
</tr>
</tbody>
</table>

HMCL: hemicellulose

Seasonal variations affect the chemical composition of the plant. Yaynesh et al., (2009) showed that during the long rainy season grass species contained crude protein levels close to the critical level suggested for maintenance and critical shortages were observed during the dry and short rainy seasons. The time of harvesting grass hay also affects their feed value. Seasonal differences, fertiliser application and harvesting time affect grain yield and chemical composition of rice straw (Shen et al., 1998). Difference
in the mineral content of wheat straw grown in different seasons also varies (Theander and Aman, 1984).

2.5. Pre treatment for delignification of lignocellulosics

The primary objective of lignocellulose pretreatment by the various industries is to access the potential of the cellulose and hemicellulose encrusted by lignin within the lignocellulose matrix (Malherbe and Cloete, 2002). For delignification purpose various physical, chemical and biological methods are used. Each method has got its significance for a specific purpose of use of biomass.

<table>
<thead>
<tr>
<th>Pre-treatments of Lignocellulosic Residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>↓ Physical</td>
</tr>
<tr>
<td>Soaking</td>
</tr>
<tr>
<td>Grinding</td>
</tr>
<tr>
<td>Pelleting</td>
</tr>
<tr>
<td>Boiling</td>
</tr>
<tr>
<td>Steaming under pressure</td>
</tr>
<tr>
<td>Gramma irradiation</td>
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<td></td>
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<td></td>
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<tr>
<td></td>
</tr>
</tbody>
</table>

2.5.1. Physical

Crop residues can be ground, soaked, pelleted or chopped to reduce particle size or can be treated with steam or X-rays or pressure cooked. Milling (cutting the lignocellulosic biomass into smaller pieces) is a mechanical pretreatment of the lignocellulosic biomass. The objective of a mechanical pretreatment is a reduction of particle size and crystallinity instead of removal of lignin. The reduction in particle size leads to an increase of available specific surface and a reduction of the degree of
polymerization thus the holocellulose becomes more accessible to use in different applications including animal feed (Hendriks and Zeeman, 2009).

During steam pretreatment the biomass is put in a large vessel and steamed with a high temperature (up to 240 °C) and pressure, applied for a few minutes. However, the impact of the change in structure of the biomass, caused by the explosion, on the digestibility is still doubted (Brownell et al., 1986). Physical treatments of crop residues have received an appreciable amount of research. Many of these treatments are not practical for use on small-scale farms, as they require machines or industrial processing. This makes these treatments, in many cases, economically unprofitable for farmers as the benefits may be too low or even negative. However, small machines to grind or chop rice straw may be feasible (Sarnklong et al., 2010).

2.5.2. Chemical

Pretreatment of the lignocellulose mainly includes acid and alkali treatments each treatment has its specific advantages and disadvantages depending upon their use. Acid pretreatment can be done with diluted or strong acids mainly sulphuric acid. Pretreatment of lignocellulose with diluted acids at ambient temperature are done to enhance the anaerobic digestibility. The objective is to solubilize the hemicellulose, and by this, making the cellulose better accessible. Chemical pretreatment with strong acids effectively increase the hydrolysis of cellulose along with the hemicellulose and lignin (Fan et al., 1982; Zheng et al., 2009).

The alkali agents can be absorbed into the cell wall and chemically break down the ester bonds between lignin, hemicellulose and cellulose, and physically make the structural fibers swollen (Lam et al., 2001). These processes enable the rumen microorganisms to attack more easily the structural carbohydrates, enhancing degradability and palatability of the rice straw. The most commonly used alkaline agents are sodium hydroxide (NaOH), ammonia (NH3) and urea (Sarnklong et al., 2010).
2.5.3. Biological

Only the removal of lignin is not the answer to enhance the nutritive quality as physical and chemical methods have their own disadvantages for the purpose. Biological deconstruction of plant cell wall has become an increasingly important research topic as the future bioeconomy depends on the supply of biomass and the feedstocks for producing bioenergy and bioproduct. Over physical and chemical pre-treatments, microbial fermentation is an inexpensive method, which takes time, but is complete and environment friendly (Abdullah et al., 2004).

2.6. Organisms involved in lignocellulose degradation

Lignocellulose is a complex substrate and its biodegradation is not dependent on environmental conditions alone, but also the degradative capacity of the different microbial populations (Waldrop et al., 2000).

Several actinomycetes and bacterial species degrade lignin including Streptomyces species, Azospirillum lipoferum, Bacillus subtilis, Bordetella kompestris, Caulobacter crescentus, Escherichia coli, Mycobacterium tuberculosum, Pseudomonas syringae, P. aeruginosa and Yersinia pestis (Alexandre and Zhulin, 2000; Arora and Sharma, 2010). The course of fungal lignocellulose degradation is most readily observable in intact dead wood. Lignocellulose biodegradation by prokaryotes like bacteria is essentially a slow process characterized by the lack of powerful lignocellulose degrading enzymes, especially lignin peroxidases. Grasses are more susceptible to actinomycete attack than wood (McCarthy, 1987). Together with bacteria, actinomycetes play a significant role in the humification processes associated with soils and composts (Trigo and Ball, 1994; Malherbe and Cloete, 2002).

2.6.1. Fungi as primary degraders

Most fungi are capable cellulose degraders. However, their ability to facilitate rapid lignocellulose degradation has attracted the attention of scientists and entrepreneurs alike. The ability of fungi to degrade lignocellulosic materials is due to their highly efficient enzymatic system. Fungi have two types of extracellular enzymatic systems; the hydrolytic system, which produces hydrolases that are responsible for
polysaccharide degradation and a unique oxidative and extracellular ligninolytic system, which degrades lignin and opens phenyl rings (Sánchez, 2009).

The degradation of wood in nature is mainly caused by fungi. The better degradative efficiency of fungi is due to their hyphal organization, which imparts them penetration capacity. Depending upon their mode of attack, the fungi are classified into three main categories as listed in Table 2.3 (Martínez et al., 2005).

(a) **Soft rot fungi:** Ascomycetes and deuteromycetes generally cause soft rot decay of wood (Daniel and Nilsson, 1998). The decayed wood has a brown, soft appearance that is cracked and checked when dry. Two forms of soft rot have been described, type I consisting of biconical or cylindrical cavities that are formed within secondary walls, while type II refers to an erosion form of degradation. The middle lamella is not attacked by type II sofrot fungi (Blanchette, 1995; 2000). *Xylariaceous ascomycetes* from genera such as *Daldinia, Hypoxylon*, and *Xylaria* have earlier often been regarded as white rot fungi, but nowadays these fungi are grouped as soft rot fungi since they cause typical type II soft rot. They primarily occur on hardwood, and weight losses up to 53% of birch wood were found within 2 months by the most efficient fungus of this group, *Daldinia concentrica* (Nilsson et al., 1989). The highest lignin loss observed was 44% at the stage when weight loss was 77% after 4 months incubation. The high concentration of guaiacyl units in the middle lamella of coniferous wood may cause the resistance to the decay by soft rot fungi. Ligninolytic peroxidases or laccases of softrot fungi may not have the oxidative potential to attack the recalcitrant guaiacyl lignin. On the other hand, syringyl lignin apparently is readily oxidized and mineralized by the enzymes of soft rot fungi (Nilsson et al., 1989). Unfortunately, ligninolytic enzymes of xylariaceous ascomycetes are not well known (Hatakka, 2001).

Microfungi or molds, i.e., deuteromycetes and certain ascomycetes that are usually thought to degrade mainly carbohydrates in soil, forest litter, and compost, can also degrade lignin in these environments. Although actinomycetes were predominant among 82 strains selected for screening ligninolytic microorganisms from forest soil; some microfungi were also identified, e.g., *Penicillium chrysogenum, Fusarium oxysporum*, and *Fusarium solani* (Hatakka, 2001).
Table 2.3 Summarized features of different types of wood decaying fungi (Martínez et al., 2005)

<table>
<thead>
<tr>
<th>Decay aspect and consistency</th>
<th>Soft rot</th>
<th>Brown rot</th>
<th>White rot</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Decay aspect and consistency</strong></td>
<td>Soft consistency in wet environments. Brown and crumbly in dry environments. Generally uniform ontogeny of wood decay</td>
<td>Brown, dry, crumbly, powdery, brittle consistency, breaks up like cubes, drastic loss of strength at initial stage of decay. Very uniform ontogeny of wood decay.</td>
<td>Bleached appearance, lighter in color than sound wood, moist, soft, spongy, strength loss after advanced decay.</td>
</tr>
<tr>
<td><strong>Host (wood-type)</strong></td>
<td>Generally hardwoods (softwoods very slightly degraded). Forest ecosystems, waterlogged woods, historic archaeological wood, utility poles.</td>
<td>Softwoods; seldom hardwoods. Forest ecosystems and wood in service.</td>
<td>Hardwood, rarely softwood</td>
</tr>
<tr>
<td><strong>Cell-wall constituents degraded</strong></td>
<td>Cellulose and hemicelluloses, lignin slightly altered</td>
<td>Cellulose, hemicelluloses. Lignin slightly modified. In some cases, extended degradation of hardwood (including middle lamella).</td>
<td>Cellulose, lignin and hemicellulose. Brittle fracture.</td>
</tr>
<tr>
<td><strong>Anatomical features</strong></td>
<td>Cell wall attack in the proximity of hyphae starts from cell lumen. Logitudinal biconical cylindrical cavities in secondary wall (Type 1). Secondary wall erosions from cell lumen (Type 2). Faculative soft rot decay by some basidiomycetes.</td>
<td>Degradation at a great distance from hyphae (diffusion mechanism). Entire cell wall attacked rapidly with cracks and clefts.</td>
<td>Cell wall attacked progressively from lumen. Erosion furrows associated with hyphae.</td>
</tr>
<tr>
<td><strong>Causal agents</strong></td>
<td>Ascomycetes (<em>Chaetomium globosum, Ustulina deusta</em>) and Deuteromycetes (<em>Alternaria alternata, Thielavia terrestris, Paecilomyces spp.</em>), and some bacteria. Some white (<em>Inonotus hispidus</em>) and brown rot (<em>Rigidoporus crocatus</em>) basidiomycetes cause facultative soft rot decay.</td>
<td>Basidiomycetes exclusively (e.g. <em>C. puteana, Gloeophyllum trabeum, Laetiporus sulphureus, Piptoporus betulinus, Postia placenta and Serpula lacrimans</em>).</td>
<td>Basidiomycetes (e.g. <em>T. versicolor, Irpex lacteus, P. chrysosporium and Heterobasidium annosum</em>) and some Ascomycetes (e.g. <em>Xylaria hypoxylon</em>).</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Basidiomycetes (e.g. <em>Ganoderma australe, Phlebia tremellosa, C. subvermispora, Pleurotus spp. and Phellinus pini</em>).</td>
<td></td>
</tr>
</tbody>
</table>
Analysis of the decayed lignin suggested that oxidative Cα-Cβ and β-O-aryl cleavages occurred during lignin degradation. Extracellular peroxidases and oxidases, e.g., laccase are also produced by microfungi, but they may not be so efficient in oxidizing lignin as those of white rot fungi (Chefetz et al., 1998; Eduardo et al., 2001).

(b) Brown rot fungi: Brown rot fungi mainly decompose the cellulose and hemicellulose components in wood, but they can also modify the lignin to a limited extent (Eriksson et al., 1990). They have been much less investigated than white rot fungi inspite of their enormous economic importance in the destruction of wood. Brown rotted wood is dark, shrink, and typically broken into brick-shaped or cubical fragments that easily break down into brown powder (Blanchette, 1995). The brown color indicates the presence of modified lignin in wood. Many brown rot fungi such as Serpula lacrymans, Coniophora puteana, Meruliporia incrassata, and Gloeophyllum trabeum are destructive to wood used in buildings and other structures (Blanchette, 1995). C. puteana and the so-called dry-rot fungus S. lacrymans, two of the most harmful fungi occurring in wood in temperate regions, prefer softwood to hardwood as substrates.

The fungal hyphae penetrate from one cell to another through existing pores in wood cell walls early in the decay process. The penetration starts from the cell lumen where the hyphae are in close contact with the S3 layer. In brown rot, the decay process is thought to affect the S2 layer of the wood cell wall first (Eriksson et al., 1990). Brown rot fungi have a unique mechanism to break down wood polysaccharides. In contrast to white rot fungi that successively depolymerise cell wall carbohydrates only to the extent that they utilize hydrolysis products in fungal metabolism, brown rot fungi rapidly depolymerise cellulose and hemicellulose, and degradation products accumulate since the fungus does not use all the products in the metabolism (Cowling, 1961; Hastrup et al., 2011).

To some extent, brown rot fungi have similar degradative capabilities and pathways as white rot fungi. Both wood decay mechanisms rely on radical formation, low pH, and the production of organic acids. They cause increased alkali solubility of lignin, and the decay is enhanced by high oxygen tension, all of which indicate a crucial
involvement of radicals, especially in the early stages of decay (Kirk, 1975; Jin et al., 1990). The production of lignin peroxidase and manganese peroxidase has been described in the brown rot fungus *Polyporus ostreiformis* (Dey et al., 1994) and laccase activity have also been detected in a brown rot fungus *Gloeophyllum trabeum* (D'Souza et al., 1996).

The initiators of both cellulose and lignin breakdown are suggested to be low molecular weight compounds that can readily diffuse from the hyphae, penetrate into wood cell and start the decay process. All models explain that brown rot decay is based on the generation of hydroxyl radicals and participation of low molecular weight non-protein compounds. They may be e.g. phenolate and other types of iron-chelating compounds, i.e. siderophores and oxalate (Evans et al., 1994; Shimada et al., 1997; Goodell, 2003; Niemenmaa et al., 2008).

Potential biotechnical applications of brown rot fungi have been studied to directly utilize crude brown rot enzyme extracts for use in the saccharification step (Lee et al. 2008), solid-state fermentation of pine sawdust for the production of cattle feed (Agosin et al., 1989) and the use of brown rotted lignin for adhesives to replace phenol formaldehyde flake board resin (Jin et al., 1990).

(c) **White rot fungi:** Basidiomycetous white rot fungi and related litter-decomposing fungi are capable of mineralizing lignin efficiently (Kirk and Farrell, 1987). Different white rot fungi vary considerably in the relative rates at which they attack lignin and carbohydrates in woody tissues. Some remove lignin more readily than carbohydrates. Many white rot fungi colonize cell lumina and cause cell wall erosion. Eroded zones coalesce as decay progresses and large voids filled with mycelium are formed. This type of rot is referred to as non selective or simultaneous rot (Blanchette, 1995). *Trametes* (syn. *Coriolus, Polyporus*) *versicolor* is a typical simultaneous-rot fungus (Eriksson et al., 1990).

Some white rot fungi preferentially degrade lignin in woody plant cell walls relatively to a higher extent than cellulose and they are called selective white rot fungi. In nature they cause white-pocket or white mottled type of rot, e.g., *Phellinus nigrolimitatus* (Blanchette, 1995; Hatakka and Hammel, 2010). There are also fungi that are able to produce both types of attack in the same wood (Eriksson et al., 1990).
Typical examples of such fungi are *Ganoderma applanatum* and *Heterobasidion annosum*. Because fungi selectively degrading lignin are considered the most promising fungi for applications in the pulp and paper industry, the search among these fungi has attained a considerable interest. However, the ratio of lignin, hemicellulose and cellulose decayed by a selected fungus can differ enormously, and even different strains of the same species, e.g., of *Phanerochaete chrysosporium* and *Ceriporiopsis subvermispora*, may behave differently on the same kind of wood (Hatakka, 2001). Several screening studies to find suitable fungi for biopulping of wood or straw have revealed fungi that, under certain conditions, degrade lignin preferentially to cellulose. Such lignin-selective fungi are, e.g., *P. chrysosporium*, *C. subvermispora* (Otjen and Blanchette, 1987), *Pycnoporus cinnabarinus* (Ander and Eriksson, 1977), *Pleurotus ostreatus* (Martínez et al., 1994), *Pleurotus eryngii* (Martínez et al., 1994), *Phlebia radiata* (Ander and Eriksson, 1977), *Phlebia tremellosus* (syn. *Merulius tremellosa*) (Ander and Eriksson, 1977; Eriksson et al., 1990), *Phlebia subserialis* (Akhtar et al., 1998), *Phellinus pini* (Eriksson et al., 1990), and *Dichomitus squalens* (Eriksson et al., 1990). The lignin-degrading systems of these fungi are important to study since they are very efficient. *C. subvermispora* may be considered as a model fungus for selective lignin degradation (Blanchette, 1995; Eriksson et al., 1990).

White rot fungi are more commonly found on angiosperm than on gymnosperm wood species in nature (Gilbertson, 1980). Usually syringyl (S) units of lignin are preferentially degraded whereas guaiacyl (G) units are more resistant to degradation. When grown on straw, transmission electron microscopy revealed that *C. subvermispora* and *P. eryngii* partially removed the middle lamella while *Phlebia radiata* apparently removed lignin from secondary cell walls (Burlat et al., 1997). In fibers, the middle lamella contains a high concentration of G lignin while secondary walls contain a high proportion of S lignin. Basic research on lignin degradation, e.g., its mechanisms, physiology, enzymology, and molecular biology, has been mainly carried out with *P. chrysosporium* (Kirk and Farrell, 1987; Eriksson et al., 1990; Gold and Alic, 1993). It was reported that both the physiological conditions for lignin degradation and the enzyme systems expressed are fungus specific and differ from those found in *P. chrysosporium*. Differences may be connected to the taxonomic position and/or ecology of the fungi, e.g., substrate specialization (hardwood, softwood, or
certain wood species, heartwood or sapwood) and the stage of degradation (Hatakka, 2001).

2.6.2. Importance of white rot fungi

Lignocellulose biodegradation by prokaryotes is of ecological significance, but lignin biodegradation by fungi, especially white rot fungi, is of commercial importance as well (Malherbe and Cloete, 2002).

In the past years, white rot fungi have been investigated to develop biotechnology for the degradation of broad-spectrum, refractory organic pollutants in the environment based on their lignin degrading enzymes. These research works have been conducted for the degradation of many wastes and environmental pollutants including dyes, pesticides, polycyclic aromatic hydrocarbons, dichlorodiphenyltrichloroethane, trinitrotoluene, polychlorinated biphenyls, chlorinated hydrocarbons, and other toxic organic compounds. White rot fungi can be applied in various environmental media (solid, liquid, and gaseous) for biodegradation. In recent years, many researchers have indicated that white rot fungi are promising microorganisms in industrial wastewater treatment (Aust, 1990; Reddy and Mathew, 2001; Gao et al., 2010).

The development of biotechnology using white rot fungi has been implemented to treat various refractory wastes and to bioremediate contaminated soils as well. Degradation of many hazardous chemicals and wastes has been demonstrated on a laboratory-scale, especially under sterile conditions. The technical challenges remain for the applications including bacterial contamination and for the scale-up of the process. The white rot fungus Pleurotus ostreatus has been applied for scaled-up bioremediation in the field. More research and development is still needed for cost-effective and sustainable applications (Gao et al., 2010).

Currently, there is interest in using white rot fungi to convert recalcitrant plant residues to value-added products for a variety of industrial applications including animal feed, fermentation and biopulping (Hatakka, 2001; Isroi et al., 2011). Lignin becomes problematic to cellulose-based wood processing, because it must be separated from cellulose at enormous energy, chemical and environmental expense. Biopulping is a solid-state fermentation process during which wood chips are treated with white rot
fungi to improve the delignification process. Biological pulping has the potential to reduce energy costs and environmental impact relative to traditional pulping operations (Breen and Singleton, 1999). Delignification is of great importance during bioethanol production. Cellulosic ethanol production is becoming feasible through research and development because lignocellulosic biomass is rich in carbohydrates (55-75% dry matter) and widely available in the form of agricultural residues (e.g. wheat straw, corn stover), energy crops (e.g. switchgrass, miscanthus) and forestry residues (e.g. poplar, pine) (Mosier et al., 2005). Various applications of white rot fungi in lignocellulose based industries are as follows (Isroi et al., 2011):

The use of lignocellulosic residues as ruminant animal feed or as a component of such feed represents one of the oldest and most widespread applications of biomass
The concept of preferential delignification of lignocellulose materials by white rot fungi has been applied to increase the nutritional value of forages (Akin et al., 1993; Chen et al., 1995; Zadrazil and Isikhuehen, 1997). This increased digestibility provides organic carbon that can be fermented to organic acids in an anaerobic environment, such as the rumen. Biological pretreatment of lignocellulose improved the nutritional value, in vitro digestibility, increase bioavailability of nutrients and decrease anti-nutritional factors (Mandebvu et al., 1999; Okano et al., 2009).

The biotechnical obstacle for improving the utilization of lignocellulose is selective removal of lignin and other aromatic constituents. P. chrysosporium, well known to be a non selective lignin degrading fungus, had little or no effect on improvement of enzymatic hydrolysis of the residues. The fungus itself consumed a large amount of readily accessible carbohydrates due to the simultaneous degradation of holocellulose and lignin (Bak et al., 2009).

Among commercially available white rot fungi, Ceriporiopsis subvermispora is highly selective in lignin degradation due to its lack of a complete cellulase system and is regarded as one of the most effective white rot fungi for biopulping (Akhtar et al., 1998; Ferraz et al., 2003). Irrespective of selective pattern, the degradation capability of white rot fungi varies among substrate types due to different chemical structures (Anderson and Akin, 2008). Modification of lignocellulosic biomass with mild chemical or physical pretreatment could facilitate the fungal degradation performance and improve delignification efficiency to a great extent (Yu et al., 2010). The addition of carbon/nitrogen sources, mineral solutions, or enzyme inducers could also improve fungal delignification processes (Shrestha et al., 2008).

Related to lignin degradation, white rot fungi face three major challenges associated with lignin structure i.e. (1) the lignin polymer is large; therefore ligninolytic system must be extra cellular, (2) lignin structure is comprised of inter unit C-C and ether bonds, therefore the degradation mechanism must be oxidative rather than hydrolytic and (3) lignin polymer is stereo irregular, therefore the ligninolytic agents must be less specific than degradative enzymes (Kirk and Cullen, 1998; Isroi et al., 2011).
White rot fungi comprise powerful lignin degrading enzymes that enable them in nature to bridge the lignin barrier and, hence, overcome the rate limiting step in the carbon cycle (Elder and Kelly, 1994). Of these, *Phanerochaete chrysosporium* is the best studied fungus. Lignin degradation by white rot fungi is an oxidative process and phenol oxidases are the key enzymes. Lignin peroxidases (LiP), manganese peroxidases (MnP) and laccases from especially white rot fungi (*P. chrysosporium*, *Pleurotus ostreatus* and *Trametes versicolor*) have been best studied. LiP and MnP oxidize the substrate by two consecutive one-electron oxidation steps with intermediate cation radical formation. Laccase has broad substrate specificity and oxidises phenols and lignin substructures with the formation of oxygen radicals. Other enzymes that participate in the lignin degradation processes are H$_2$O$_2$-producing enzymes and oxidoreductases, which can be located either intra or extracellularly (Kapoor et al., 2005).

2.7. Enzymology of lignocellulosic degradation

Lignocellulolytic enzymes producing fungi are widespread. Biomass degradation by these fungi is performed by complex mixture of cellulases, hemicellulases and ligninases, reflecting the complexity of the enzyme system and substrate.

2.7.1. Hemicellulases

Several different enzymes are needed to hydrolyze hemicelluloses, due to their heterogeneity. The complete hydrolysis of hemicellulose into monosaccharides requires the concerted action of xylanases, galactanases, mannanases, xylosidase, galactosidase and mannosidase (Cullen and Kersten, 1992).

Xylan is the most abundant component of hemicellulose contributing over 70% of its structure. Xylanases (Endo-β-1,4-xylanase; EC: 3.2.1.8) are able to hydrolyze β-1, 4 linkages in xylan (Figure 2.8) and produce oligomers which can be further hydrolyzed into xylose by β-xylosidase (Ustinov et al., 2008; Dashtban et al., 2009).
Birchwood xylan contains 94% of carbohydrate as xylose (more than 90% is in the form of soluble xylan), which is an ideal substrate for standardizing the activity of endoxylanase. Xylanase assay is usually done using birchwood xylan, which is mainly present as methyl-glucuronoxylan as substrate and contains 90% xylan (Bailey et al., 1992).

2.7.2. Cellulases

Hydrolysis of the β-1,4-glycosidic bonds in cellulose can be achieved by many different enzymes known as cellulases which use two different catalytic mechanisms, the retaining and the inverting mechanisms. In both mechanisms, two catalytic carboxylate residues are involved and catalyze the reaction by acid-base catalysis (Dashtban et al., 2009). Three classes of hydrolytic cellulases are recognized on the basis of substrate specificity (Deobald and Crawford 1997).

(i) Endo 1,4-β-glucanases (EG) (EC 3.2.1.4, endocellulase): cleave randomly at 1,4-β-linkages within the cellulose chain. Endoglucanases are also referred to as carboxymethylcellulases (CMCase), named after the artificial substrate used to measure the enzyme activity. The enzyme initiates cellulose breakdown by attacking the amorphous regions of the cellulose, making it more accessible for cellbiohydrolases by providing new free chain ends. This has been shown by the effect of the enzyme on CMC and amorphous cellulose.

(ii) Exo 1,4-β-glucanase (EC 3.3.1.91, exocellulase) (Exo 1,4-β-D-glucan cellbiohydrolases, CBH) releases both glucose and celllobiose from the nonreducing
ends of cellulose chains. It is generally estimated using Whatman filter paper as a substrate and expressed as filter paper activity (FPAase).

(iii) 1,4-β-glucosidases (EC 3.2.1.21) hydrolyse cellobiose to glucose, and celllobionic acid to glucose and gluco-nolactone. Thus, the activity can be measured using cellobiose as a substrate.

2.7.3. Lignin modifying enzyme system

The ligninolytic system of white rot fungi is not homogenous. Different white rot fungi have been shown to possess one or more enzymes. Ligninolytic enzymes consist of mainly lignin peroxidases (LiPs; E.C.1.11.1.14), manganese peroxidases (MnPs; E.C.1.11.1.13) and versatile peroxidases (VPs; E.C.1.11.1.16) and laccases (E.C.1.10.3.2). Some or all of these enzymes and their isozymes can be produced by a number of wood rotting fungi including white rot basidiomycetes, brown rot basidiomycetes, and soft rot ascomycetes/deuteromycetes fungi (Hatakka, 2001; Singh and Chen, 2008).

**Laccase:** Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) represents a family of copper-containing polyphenol oxidases and usually called multicopper oxidases. The first laccase, from the Japanese lacquer tree, *Rhus vernicifera*, was described in 1883 (Yoshida, 1883). Subsequently, laccases and laccase-like proteins have been described in plants, fungi, arthropods and bacteria (Rodgers *et al.*, 2010). Laccases are glycoproteins with molecular weight of about 60 kDa, while it ranges between 50-130 kDa, (Morozova *et al.*, 2007).

Laccases catalyze monoelectronic oxidation of substrate molecules to corresponding reactive radicals with the assistance of four copper atoms that form catalytic core of the enzyme, accompanied with the reduction of one molecule of oxygen to two molecules of water and the concomitant oxidation of four substrate molecules to produce four radicals (Riva, 2006). However, all substrates cannot be directly oxidized by laccases, either because of their large size which restricts their penetration into the enzyme active site or because of their particular high redox potential. To overcome this hindrance, suitable chemical mediators are used which act
as intermediate substrate for laccase, whose oxidized radical formed are then able to interact with high redox potential substrate targets (Figure 2.9) (Riva, 2006).

**Figure 2.9** Schematic representation of laccase catalyzed redox cycle for substrate oxidation in the presence of chemical mediator (Riva, 2006; Arora and Sharma, 2010)

**Lignin Peroxidase (LiP):** Lignin peroxidase was first discovered based on the $\text{H}_2\text{O}_2$ dependent $\alpha$-$\beta$ cleavage of lignin model compounds and subsequently shown to catalyze the partial depolymerization of methylated lignin *in vitro* (Tien and Kirk, 1984). LiPs are glycoproteins with MWs estimated from 30 to 46 kDa (Cullen and Kersten 2004).

Lignin peroxidase catalyzes a variety of oxidations, all of which are dependent on $\text{H}_2\text{O}_2$. These include $\alpha$-$\beta$ cleavage of the propyl side chains of lignin and lignin models, hydroxylation of benzylic methylene groups, oxidation of benzyl alcohols to the corresponding aldehydes or ketones, phenol oxidation, and even aromatic ring cleavage of nonphenolic lignin model compounds (Tien and Kirk, 1984; Niladevi, 2009). Hydrogen peroxide oxidizes resting enzyme by two electrons to give Compound I enzyme intermediate. Compound I oxidizes aromatic substrates by one electron to give Compound II (a one-electron oxidized enzyme intermediate) that can again oxidize substrate to return the enzyme to resting state.

\[
\text{LiP} + \text{H}_2\text{O}_2 \rightarrow \text{Compound I} + \text{H}_2\text{O}
\]
\[
\text{Compound I} + \text{S} \rightarrow \text{Compound II} + \text{S}^{+}\bullet
\]
\[
\text{Compound II} + \text{S} \rightarrow \text{LiP} + \text{S}^{+}\bullet
\]
(LiP represents the ferric state resting lignin peroxidase and S represents an aromatic substrate)

Although the assortment of reactions is very complex, the initiation of these reactions is simple. Lignin peroxidase oxidizes the aromatic substrates by one electron;
the resulting aryl cation radicals degrade spontaneously via many reactions dependent on the structure of the substrate and on the presence of reactants (Cullen and Kersten, 1992).

**Manganese peroxidases (MnP):** Manganese peroxidases are the most abundant group of extracellular ligninolytic enzymes in white rot fungi (Gold and Alic, 1993, Hatakka, 1994). This heme containing glycoprotein was discovered in *Phanerochaete chrysoporium* almost 20 years ago. The MW of MnP ranges from 38 to 62.5 kDa, but most purified enzymes have MWs around 45 kDa (Hofrichter et al., 2002).

The principle function of the enzyme is to oxidize Mn\(^{2+}\) to Mn\(^{3+}\), using H\(_2\)O\(_2\) as oxidant. Activity of the enzyme is stimulated by simple organic acids which stabilize the Mn\(^{3+}\), thus producing diffusible oxidizing chelates (Glenn et al., 1986). Manganese peroxidase enzyme intermediate are analogous to other peroxidases. Native manganese peroxidase is oxidized by H\(_2\)O\(_2\) to Compound I, which can then be reduced by Mn\(^{2+}\) and phenols to generate Compound II. Compound II then is reduced back to a resting state by Mn\(^{2+}\), but not by phenols (Wariishi et al., 1989). Therefore Mn\(^{2+}\) is necessary to complete the catalytic cycle and shows saturation kinetics (Wariishi et al., 1988). Kinetic studies with Mn\(^{2+}\) chelates support role for oxalate in the reduction of manganese peroxidase Compound II by Mn\(^{2+}\) (Kishi et al., 1994).

\[
\text{Native (ferric) peroxidase} + \text{H}_2\text{O}_2 \rightarrow \text{Compound I} + \text{H}_2\text{O} \\
\text{Compound I} + \text{Mn}^{2+} \rightarrow \text{Compound II} + \text{Mn}^{3+} \\
\text{Compound II} + \text{Mn}^{2+} \rightarrow \text{Native (ferric) peroxidase} + \text{Mn}^{3+}
\]

Aeration and consequent oxygen availability are extremely important in studying the physiology of fungal growth, in particular lignin biodegradation by white rot fungi (Kerem and Hadar, 1993).

As shown in Figure 2.10, laccases or ligninolytic peroxidases (LiP, MnP, and VP) produced by white rot fungi oxidize the lignin polymer, thereby generating aromatic radicals (a). These evolve in different non-enzymatic reactions, including C4-ether breakdown (b), aromatic ring cleavage (e), Cα-Cβ breakdown (d), and demethoxylation (e).
Figure 2.10 A scheme for lignin biodegradation including enzymatic reactions and oxygen activation (Martínez et al., 2005).

The aromatic aldehydes released from Cα-Cβ breakdown of lignin, or synthesized de novo by fungi (f, g) are the substrate for $\text{H}_2\text{O}_2$ generation by ary1-alcohol oxidase (AAO) in cyclic redox reactions involving also ary1-alcohol dehydrogenases (AAD). Phenoxy radicals from C4-ether breakdown (b) can repolymerize on the lignin polymer
(h) if they are not first reduced by oxidases to phenolic compounds (i), as reported for AAO. The phenolic compounds formed can be again reoxidized by laccases or peroxidases (j). Phenoxy radicals can also be subjected to Cα-Cβ breakdown (k), yielding p-quinones. Quinones from g and/or k contribute to oxygen activation in redox cycling reactions involving quinone reductases (QR), laccases, and peroxidases (l, m). This results in reduction of the ferric iron present in wood (n), either by superoxide cation radical or directly by the semiquinone radicals, and its reoxidation with concomitant reduction of H₂O₂ to hydroxyl free radical (OH·) (o). The latter is a very strong oxidizer that can initiate the attack on lignin (p) in the initial stages of wood decay, when the small size of pores in the still-intact cell wall prevents the penetration of ligninolytic enzymes. Then, lignin degradation proceeds by oxidative attack of the enzymes described above. In the final steps, simple products from lignin degradation enter the fungal hyphae and are incorporated into intracellular catabolic routes (Martínez et al., 2005).

2.8. Degradation of agricultural residues for animal feed bioprocessing

Delignification has potential in variety of industrial fields including pulp and paper, textile, and food industries. Biodelignification mainly based on ligninolytic enzyme systems, is advantageous over physical and chemical treatments as enzymes are biodegradable catalysts and specific in action, and enzymatic reactions are carried out in mild conditions.

Cellulose is the most important source of carbon and energy in a ruminant’s diet, although the animal itself does not produce cellulose-hydrolyzing enzymes (Czerkowski, 1986). Rumen microorganisms utilize cellulose and other plant carbohydrates as their source of carbon and energy. Thus, the microorganisms convert these complex carbohydrates in simple sugars and a large amount of acetic, propionic and butyric acids, which the higher animal can use as its energy and carbon sources (Colberg, 1988).
In order to increase digestibility of lignocellulose, biological methods can be used. Biodelignification of such agricultural lignocellulosics not only enhances the digestibility of the feed but also improves their nutritional value. These methods are mostly based upon the decomposition of lignin after the splitting of the cellulose–lignin complex (Figure 2.11). The main problem of biological upgrading of lignocellulose is to select microorganisms capable of degrading the lignin selectively. Suitable microorganisms should metabolize the lignin efficiently and selectively avoiding cellulose degradation under the fermentation conditions (Villas-Boás et al., 2002).

Fermentation processes may be divided into two systems: submerged fermentation (SmF), which is based on the microorganisms cultivation in a liquid medium containing nutrients, and solid state fermentation (SSF), which consists of the microbial growth and product formation on solid particles in the absence (or near absence) of water; however, substrate contains the sufficient moisture to allow the microorganism growth and metabolism (Pandey, 2003). In recent years, SSF has received more interest from researchers since several studies have demonstrated that this process may lead to higher yields and productivities or better product characteristics than SmF. In addition, due to the utilization of low cost agricultural and agroindustrial residues as substrates, capital and operating costs are lower as compared to SmF. The low water volume in SSF has also a large impact on the economy of the process mainly due to simplicity, cost effectiveness, maintenance requirements, smaller fermenter-size,
reduced downstream processing, reduced stirring and lower sterilization costs (Hölker and Lenz, 2005). The main drawback of this type of cultivation concerns the scaling-up of the process, largely due to heat transfer and culture homogeneity problems (Di Luccio et al., 2004). However, research attention has been directed towards the development of bioreactors that overcome these difficulties. At this stage, engineering aspects come into play and the success of scale-up will depend on bioreactor design and operation (Lonsane et al., 1992; Martins et al., 2011).

The combination of solid-state fermentation (SSF) technology with the ability of white rot fungi to selectively degrade lignin has made possible industrial-scale implementation of lignocellulose-based biotechnologies. SSF offers the advantages of a robust technology and outperforms conventional fermentation technologies. These advantages make SSF an attractive technology for environmental problems where money and expertise are limited. For the conversion of lignocellulosic biomass into more nutritive feed, delignification in SSF is a better choice. Basic key features including selective ligninolysis, enhancement in protein, amino acid, IVD and easy harvesting of residues make SSF a preferable technique over submerged one.

As suggested by Sarnklong et al., (2010), using ligninolytic fungi, including their enzymes, may be one potential alternative to provide a more practical and environmental-friendly approach for enhancing the nutritive value of straw. The cost of exogenous enzymes is at present too high to be applied by small holder farms, but this may change in the future.

2.9. Effect of supplements on fungal degradation of lignocellulosics

The lignocellulolytic enzyme system and lignocellulosic degradation profile of the fungus depends upon the nutritional and physical conditions. Low quality roughages such as cereal straw and stover are generally high in fibre but low in key nutrients such as nitrogen and minerals. Energy supplementation has been reported as being variably successful in enhancing digestibility (Fonseca et al., 2001; Migwi et al., 2011). The application of ligninolytic fungi in combination with chemical pretreatments to straw may be an alternative way to shorten the period of the incubation and decrease the amount of chemicals for lignocellulosic degradation (Sarnklong et al., 2010).
Scientists have reported the significance of carbon nitrogen ratio during fungal degradation of lignocellulosic residues. Enhancement in ligninolysis and minimizing the polysaccharide loss has been reported by addition of synthetic nitrogen sources or some complex organic supplements, while during some studies nitrogen starvation conditions were responsible for more ligninolysis (Reid, 1983; Commanday and Macy, 1985). Among different inorganic supplements the addition of ammonium salts in the form of chloride, nitrate, sulphate, tartrate etc. have been used frequently, which showed different results depending upon the substrate, fungal species and degradation conditions. Similarly urea, peptone, yeast extract, malt extract, soybean meal, casein, albumen hydrolysates etc. have been used as complex organic nitrogen rich supplements (Al-Ani and Smith, 1986).

A lot of work has been done on the production of different lignocellulolytic enzymes by white rot fungi and its optimization studies; but only a scant literature is available on the optimization of biodelignification of agro-residues and enhancement in digestibility (Basu et al., 2002). Traditional method of single factor optimization by maintaining other factors at constant level does not reveal the cumulative effect of all the factors involved. The single variable optimization methods are tedious and the interaction between different factors is overlooked. Some statistical methodologies can be applied for better optimization studies. Response surface methodology (RSM) is a powerful technique for testing multiple variables simultaneously, which also provide the interactive effect between different variables (Jeya et al., 2009; Wejse et al., 2003). In RSM studies, limited number of experiments is required to be performed and one is able to determine accurate optimum values of test variables (Adinarayana and Ellaiah, 2002).
2.10. Enhancement in nutritional quality

According to Alves De Brito et al., (2003), evaluation of the nutritional quality of forage requires the detailed analysis of the composition of its cell wall. The digestibility of cell wall is influenced by both the content and physical characteristics of wall polysaccharides such as degree of crystallinity and polymerization (Fritz et al., 1990).

2.10.1. In vitro digestibility

Digestibility measured by in vitro methods gives a close idea about the quality of feed (Goering and Van Soest, 1970) as this provides a quick and precise prediction of in vivo digestibility in ruminants. The in vitro procedure does a better job of prediction than chemical composition because it accounts for all factors affecting digestibility, whether known or unknown, which is not possible by present chemical methods (Garcia et al., 2003).

The two stage in vitro procedure developed by Tilley and Terry (1963) is the most reliable laboratory method for predicting the digestibility of a wide range of forages. It can predict in vivo digestibility with a lower error than any chemical method (Minson, 1982) and has been widely accepted throughout the world for measuring the digestibility of feeds (Minson, 1990). Many fungi produce cellulase, hemicellulase and other enzymes that degrade forage carbohydrates. Jones and Hayward (1973), showed that a commercially available fungal cellulase could be used to predict forage digestibility with an accuracy similar to that achieved with the method of Tilley and Terry. Unfortunately, these cellulase preparations are relatively expensive and not readily available in less developed countries. Consequently, enzymatic methods have generally received less attention than the procedure of Tilley and Terry.

The first stage of the Tilley and Terry (1963), technique simulates conditions in the reticulorumen and requires an inoculum of rumen micro-organisms obtained from sheep or cattle fitted with a rumen fistula. The use of fistulated animals for this purpose has been criticized on ethical grounds, but there are also many practical reasons for reducing the need for fistulated animals in nutritional studies including (a) the production of animals with rumen fistulae requires special surgical skills, (b) fistulated animals need special care to ensure that the fistula is kept free of any infection and (c) a
uniform diet must be fed if the inoculum is to have constant activity. These conditions are often difficult or impossible to achieve in the humid tropics and in less-developed countries (Akhter et al., 1999). It is also difficult to handle an animal for practical use in a typical microbiology lab.

One method of overcoming the need for rumen-fistulated animals is to use freshly voided faeces from sheep as the source of inoculum (El Shaer et al., 1987). Akhter et al., (1999), concluded that bovine faeces have the potential as an alternative to rumen liquor from rumen-fistulated sheep when estimating digestibility using the in vitro technique. Further, the method involving the use of enzymes like cellulase is relatively expensive, while the one using faecal inoculum for determining the digestibility is comparatively cheaper and easy laboratory method and equally effective as that of using rumen fluid (Dhanoa et al., 2004).

2.10.2. Crude protein

Feed protein generally refers to crude protein. Crude protein is termed “crude” because it is not a direct measurement of protein but is an estimation of total protein based on nitrogen (N x 6.25 = crude protein), which assumes 16 g of N/100 g of protein in feeds. Crude protein includes true protein and non-protein nitrogen (NPN) such as urea nitrogen and ammonia nitrogen. The crude protein value provides no information about amino acid composition, intestinal digestibility of the protein, or its rumen degradability (Garcia et al., 2003; Rotz, 2004).

2.10.3. Amino acids

Proteins are composed of amino acids, which are required for the maintenance, growth, and productivity of animals. A total of about 22 amino acids have even identified of which the animal can synthesise about half; which are called non-essential amino acids. However the others cannot be synthesised and must be provided in the diet. These are called essential amino acids. These amino acids are the most important nutritionally because limiting these will affect the growth and development of the animal. Supplemental amino acids can be added to feedstuff to increase efficiency of animal production and achieve a least cost feed formulation. Analysing the amino acid composition of feedstuffs ensures that nutritionists provide a more precise feed formulation (Rotz, 2004).
Ninhydrin method was introduced for quantitative determination of amino acids in the late 1940s. The method was originally developed for chromatographic elution from amino acid analyzer (Moore and Stein, 1948), this method has also been adapted for determination of amino group containing compounds in foods as well as various types of samples (Hurst et al., 1995; Panasiuk et al., 1998). Recently, the applications of ninhydrin method for the determination of amino compounds in pharmaceutical products (Frutos et al., 2000) and quantification of collagen-like polymer in microbial cell lysate (Yin et al., 2002) have also been reported, indicating a continued popularity of this method (Sun et al., 2006).

Although instrumental methods such as amino acid analyzer and HPLC are currently used for determining compounds containing amino group, the simple and convenient ninhydrin method still possess several advantages because no expensive equipment is required, and it is suitable for the routine analysis of large numbers of samples.

The modifications suggested by Sun et al., (2006) make ninhydrin method even more convenient, less expensive, and less time consuming for quantification of compounds containing amino group. Less expensive sodium hydroxide/acetic acid buffer or simple salt such as sodium acetate could be used in the ninhydrin method, and shorter heating time (10 min) could be achieved. Amino acids produce the typical purple-blue or yellow colour during ninhydrin reaction, which can easily be measured colorimetrically. The method has also been used for the quantitative analysis of amino acids in a variety of agricultural residues (Friedman, 2004).

2.10.4. Antioxidant properties

Vitamin E, vitamin C, carotenoids and some trace minerals are important antioxidant components of animal feed and their role in animal health and immune function are indispensable (Roeder, 1995). Free radicals are mainly reactive oxygen species (ROS) and reactive nitrogen species (RNS) and include not only the oxygen or nitrogen radicals, but some non radical reactive derivatives of oxygen and nitrogen. Free radicals like superoxide anion (O$_2^-$), alkoxyl (RO.) radical, nitric oxide (NO), hydrogen peroxide (H$_2$O$_2$), peroxy radical (ROO.) and hypochloride (HOCl) are constantly produced during normal physiological metabolism in tissues. Biologically important
molecules such as DNA, proteins, lipids and carbohydrates are damaged by these free radicals (Bellomo, 1991). Under normal conditions the deleterious effects of ROS/RNS are countered by the body’s antioxidant defenses, which are contributed through dietary intake of key nutrients (e.g. vitamins and trace minerals). Antioxidants serve to stabilize these highly reactive free radicals, thereby maintaining the structural and functional integrity of cells. Thus, along with nutritive feed the antioxidants are very important for the immune system and health of the animals (Chew, 1995).

A variety of polyphenols contributes to the antioxidant potential of feed. Cereal straw contains lignin, which are complex phenolic polymers and exhibit a very poor solubility. This may limit their reactivity with the radicals responsible for the oxidation and subsequently limit their protecting effect compared to that of synthetic antioxidants. The delignification comprises the cleavage of covalent linkages of the lignin, which results into the formation of low molecular weight units holding a great value in antioxidant enhancement (Pouteau et al., 2003). Thus, degradation of lignin enhances smaller phenolic units and holds the potential to upgrade the quality of lignocellulosic residue by enhancing its antioxidant property.

2.11. Toxicity of fermented animal feed

Different workers have used white rot fungi to upgrade the nutritive quality of lignocellulosic residues. Apparently, no case has been reported for the pathogenicity of these fungi towards human and animal species. The potential for biological hazard is low for the microbially converted feeds so far evaluated (Sinskey and Batt, 1987; Banerjee et al., 2000). During a recent study on fungal fermented wheat straw (Sharma et al., 2011), different mycotoxins (aflatoxins) were observed but the levels of the toxins in all the diets were far below to the permissible levels (20 ppb) in the feeds meant for immature animals (Food and Drug Administration, USA (USFDA)) and poultry (Bureau of Indian Standards (BIS)). Nevertheless, this hazard has to be continually evaluated by various biological studies when a new microbially fermented product is proposed.

Tests for toxicity of the organism and the treated biomass as well as for improved performance in large-scale animal trials are necessary to further evaluate the potential of solid state fungal treatment of lignocellulose (Akin et al., 1993).
Mycotoxins are secondary metabolites produced by fungal species mainly including *Aspergillus, Penicillium, Fusarium, Alternaria* and *Stachybotrys* spp. During the growth of the moulds on food and feed stuff the toxins may be produced. A number of bioassays using cell culture techniques have been described for the toxicological characterization of mycotoxins. A colorimetric cell culture assay using 3-(4,5-dimethylthiazolyl-2)-2,5 diphenyltetrazolium bromide (MTT) is an important measure of cellular toxicity, firstly used by Mosmann (Mosmann, 1983; Zödl *et al.*, 2005). MTT can reflect the sensitivity of live cells to extrinsic stimulation; therefore, it also has an important value to assess cell viability. Earlier, to detect toxin effects in *in vitro* cell models, the (MTT) dye reduction assay has been shown to be an effective indicator of *Campylobacter jejuni* toxicity (Gilbert and Slavik, 2004).

As reported by Zadrazil, (2000), some fungi decompose lignin and other substrate components, but *in vitro* digestibility decreases. This may be due to toxicity for the rumen microorganisms of substrate extracts that are used for the determination of *in vitro* digestibility. This concept is also helpful to know about the toxicity of fermented feed towards rumen microflora.

Some mycotoxins especially Aflatoxin B1 was reported to be highly mutagenic in the *Salmonella typhimurium* ("Ames test") system (Ciegler and Bennett, 1980). Ames test is well known for the assessment of mutagenic activity (Maron and Ames, 1983). Feed extract against *S. typhimurium* was used to evaluate the mutagenic activity of extract.

On the basis of the literature, it can be concluded that white rot fungi can delignify the lignocellulosic residues using their well developed ligninolytic enzyme system. Break down of lignin hemicellulose matrix also enhances the susceptibility of cellulosic matter by providing better exposure to enzymes. Thus, the study was carried out to delignify the cereal residues by white rot fungi and to monitor their impact on *in vitro* digestibility. As the chemical composition of the substrate affects the degradability, experiments involving degradation of lignocellulosic residues collected from different geographic locations, use of nitrogen rich supplements and other optimization studies were carried out. Finally, the experiment was scaled up from 5g to 200g batches to check the efficiency of the process. The success of the experimentation has also been
subjected to evaluation by working out the toxicity, if any, of the delignified biomass or feed. The process also involved the production of different lignocellulolytic enzymes, which can be harvested simultaneously for industrial uses to provide added advantage.