Chapter 2

REVIEW OF LITERATURE

2.1 Pleurotus

Pleurotus (oyster) mushrooms belong to class Basidiomycetes and family Tricholomataceae (Martinez, 1998; Syed et al., 2009). They are commonly called as ‘Dhengri’ in India, and have about 40 well-recognized species, out of which 12 species are cultivated in different parts of country (Syed et al., 2009). Pleurotus mushroom is fleshy and normally oyster shell-like (about 5 - 20 cm in diameter), and their colour can be white, cream, yellow, pink, brownish, or dark gray (Martinez, 1998).

Pleurotus mushrooms show the typical life cycle of Basidiomycetes while growing on lignocellulosic substrates. Life cycle has two stages: the vegetative stage and the reproductive stage. Generally, some kinds of stimuli are needed for the shift from mycelial (vegetative) growth to the fruit body formation (reproduction) phase. When environmental conditions are appropriate (temperature, light, relative humidity), the mycelium will differentiate into fruit bodies (Martinez, 1998).

2.2 Lignocellulosic biomass

Lignocellulosic biomass mainly consists of lignin, cellulose, hemicellulose, pectin and other components (Bruce, 1987). Crop residues are high in cellulose,
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hemicellulose and lignin, but low in pectin (Parveen et al., 2009). Chemical composition of some common lignocellulosic materials is given in table 2.1.

Table 2.1 Chemical composition of some common lignocellulosic biomass

<table>
<thead>
<tr>
<th>Components</th>
<th>Corn stover</th>
<th>Rice straw</th>
<th>Wheat straw</th>
<th>Switch grass</th>
<th>Sugarcane bagasse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulose (%)</td>
<td>15</td>
<td>35</td>
<td>30</td>
<td>45</td>
<td>40</td>
</tr>
<tr>
<td>Hemicellulose (%)</td>
<td>35</td>
<td>25</td>
<td>50</td>
<td>30</td>
<td>24</td>
</tr>
<tr>
<td>Lignin (%)</td>
<td>8</td>
<td>12</td>
<td>20</td>
<td>12</td>
<td>25</td>
</tr>
<tr>
<td>Pectin (%)</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>


Lignocellulosic biomass are generally considered as a low-grade domestic fuel and burnt negligently or thrown away. About 50% of this biomass source is burned for cooking and heating and left or combusted directly on the land so that a lot of crop straw and stalk resources are lost and serious environmental pollution occurs. Agricultural residues represent an abundant, inexpensive, and readily available source of renewable lignocellulosic biomass (Ruigang et al., 2003). Therefore the development of a biotechnological process to convert agro-industrial wastage up to a non-harmful as well as useful level will have environmental importance (Zadrazil, 2000; Peixoto-Nogueira et al., 2009). Increased awareness
of global deforestation has raised the demand of alternatives to wood as a raw material for pulp and paper. Hence, rather than destroying this valuable raw material, using it for value-added purposes would be more environmentally and economically beneficial (Ruigang et al., 2003).

2.3 Components of lignocellulosic biomass

2.3.1 Cellulose

Cellulose is the principle component of lignocellulosic biomass and its concentration ranges from 40 to 50 % of dry weight. Cellulose is a homopolysaccharide composed of repeating β – D - glucopyranose units (Bishnu et al., 2011). The degree of polymerization and crystallinity of cellulose varies from species to species and this is shown to have a significant impact on hydrolytic process (acidic and enzymatic) (Zhang and Lynd, 2004). Cellulose is one of the most important natural polymers in terms of its annual production and in its industrial applications (Ruigang et al., 2003). Crystalline structure of cellulose also is highly resistant to breakdown by enzymes (Langer et al., 1980, Henics, 1987).

2.3.2 Hemicellulose

Hemicellulose is less complex, its concentration in lignocellulosic biomass is 25 to 30 % and it is easily hydrolysable to fermentable sugars (Saha et al., 2007). Hemicellulose is a heteropolysaccharide composed of pentoses (D - xylose and D - arabinose), hexoses (D - mannose, D - glucose and D - galactose) and sugar acids (Bishnu et al., 2011). Softwood hemicellulose mainly contains mannose as a major constituent whereas hardwoods mainly contain Xylans (Balan et al., 2009).
2.3.3 Lignin

Lignin is the third major component of lignocellulosic biomass and its concentration ranges for 20 to 35%. It is a complex polymer of phenyl propane units (coumaryl, coniferyl and sinapyl alcohol) (Bishnu et al., 2011). Lignin acts as cementing agent and an impermeable barrier for enzymatic attack (Howard et al., 2003). Lignin provides plants with the structural support and impermeability they need as well as resistance against microbial attack and oxidative stress (Bishnu et al., 2011). These properties of lignin may be attributed to its amorphous nature, water insolubility and optical inactivity. The latter properties also make it tough to degrade (Fengel and Wegener, 1984). Lignin forms a lignocellulosic complex with the carbohydrates and proteins (Oziel et al., 2008).

2.3.4 Pectin

In some biomass types, such as sugar beet pulp and citrus peel, pectin can also comprise a significant portion of the lignocellulose structure (Grohmann and Baldwin, 1992; Micard et. al., 1996; Peterson et. al., 2008). In lignocellulosic substrates, pectin interacts with lignin, hemicellulose, and cellulose. The lignin – hemicellulose - pectin complex forms one of the most stringent seals around cellulose (Bishnu et al., 2011). Hence the degradation of pectin is necessary for the disintegration of these cell wall components (Edwards et al., 2011).

2.4 Recalcitrance of lignocellulosic biomass

The digestibility of lignocellulosic materials is very low (Kinfemi et al., 2009). Cell wall lignification of lignocellulosic materials is the major factor that limits the availability of cell wall structural carbohydrates (cellulose and hemicellulose) for utilization (Kerley et al., 1988). Lignin forms a lignocellulolic complex with the carbohydrates and proteins (Oziel et al., 2008). Crystalline structure of
cellulose also is highly resistant to breakdown by enzymes (Langar et al., 1980; Henics, 1987).

Utilization of lignocellulosic biomass as a carbohydrate source for glucose and ethanol production, and as a metabolic energy source in ruminant feeds, has been severely hampered by the low efficiency with which organisms and enzymes are able to convert the polysaccharide portion of the residue into monomeric sugars. This is again attributed to the lignin component of the cell wall and its association with other cell wall polysaccharides (Gould, 1989; Sun et al., 2000).

2.5 Advantages of *Pleurotus* cultivation over other fungi with respect to lignocellulosic substrate degradation

Several studies have addressed the degradation of lignocellulosics by direct cultivation of fungi (Tellez et al., 2008; Elisashvili et al., 2008). The white-rot basidiomycetes are strong decomposers of lignocellulosic wastes, when compared to other fungi, due to their capability to synthesize the relevant and unique oxidative and hydrolytic network of lignocellulolytic extracellular enzymes (Maganhotto, 2005; Eichlerova et al., 2006). *Pleurotus*, a versatile genus of white-rot basidiomycetes fungi, is well known for its complexity of enzymatic system and prominent lignocellulolytic property, and can colonize a wide range of natural lignocellulosic wastes (Elisashvili et al., 2006; Naraian et al., 2010).

The cultivation of oyster mushrooms offers one of the most feasible and economic method for the bioconversion of agro-lignocellulosic wastes (Rajarathnam and Bano, 1989; Cohen et al., 2002). *Pleurotus* spp. have a unique ability to produce xylanase (Elisashvili et al., 2008), carboxy methyl cellulase, β - glucosidase, β – xylosidase, and extracellular lignocellulolytic enzymes including laccase and lignin peroxidase (Stajic et al., 2006).
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2.6 Cultivation of Pleurotus spp. on lignocellulosic substrates

Cultivation of the oyster mushroom has increased significantly throughout the world during the last few decades (Chang, 1999; Royse, 2002). In the recent times, the cultivation of Pleurotus spp. had excelled next to Agaricus bisporus (Erkel, 1992; Chang and Miles, 1991). At present, Pleurotus spp. is the second most important cultivated mushroom in terms of world production. It is a very simple and low cost production technology and gives consistent growth with high biological efficiency (Rajarathnam and Bano, 1989; Cohen et al., 2002). Cultivation is with a slight variation in the range and combination of the substrates based on their availability and cost in the respective region (Royse, 1985; Schmidt, 1986). New technologies and production techniques are being constantly developed as the number of required controllable environment parameters increases (Holker et al., 2004; Syed et al., 2009). A good quality spawn is essential for the stable production of mushrooms, because, spawn quality enhances mushroom yields (Zhang et al., 2002). Modern methods of spawn preparation use cereal grains (e.g. wheat, millet, rye), which are sterilized in glass jars or polypropylene plastic bags, inoculated with a selected strain, and incubated at appropriate temperatures for complete colonization.

Pleurotus spp. is commonly cultivated on pasteurized wheat or rice straw, but it can be also cultivated on a wide variety of lignocellulosic substrates, enabling it to play an important role in managing organic wastes whose disposal is problematic. Rice straw was appeared to be the best substrate for Pleurotus mushroom cultivation when compared to banana leaves, maize stover, corn husks, rice husks and elephant grass (Obodai et al., 2003). If properly sterilised, several substrates can be made suitable for cultivation - wood shavings, banana pseudo stem, waste paper, diverse plant leaves, sawdust mixtures, pulp mill sludges etc. (Gregori, 2007).
Prepared substrates are homogeneously inoculated with the spawn, either by hand or mechanically, at the rate of 0.5 - 3 % of fresh substrate weight. The spawned substrate is placed in a variety of containers. Different containers like bottle, tray, jar, grid-frame, wall-frame, horizontal trays, shelves, vertical plastic sacks, pressed rectangular blocks and others can be used for mushroom cultivation (Stamets, 2000). In practice, the most used are bag, bottle and shelf cultivation (Choi, 2003). Containers are placed inside growing rooms for incubation. Light, ventilation and watering are increased in the growing rooms, after complete colonization of the substrate by the mushroom mycelium (15 - 40 days), to promote fruiting (Martinez, 1998).

Carbon dioxide, temperatures, composition of the growing substrate etc. are some of the factors which affect the growth and fruiting of mushrooms. Cellulose / lignin ratios in substrates were correlated to mycelial growth rates and mushroom yields of Pleurotus spp. (Diamantopoulou, 2001). Production of fruit bodies varies according to each species, spawn quality, substrate quality, environmental conditions (temperature, light, relative humidity, concentration of $O_2 / CO_2$), and impact of pests (flies, mites) and diseases (fungi, bacteria, viruses). Average biological efficiencies (yield of fresh mushrooms as a percentage of the dry weight of substrate at spawning) reported from diverse substrates range from 35 - 159 %, considering a whole production cycle of about 70 - 80 days (Kong, 2004).

### 2.7 Production of extracellular enzymes during growth of Pleurotus spp. on lignocellulosic substrates

During the colonization of the lignocellulosic substrate, Pleurotus spp. converts easily digestible carbohydrates into simpler sugars by a process known as primary metabolism (Yamakama et al., 1992; Oziel et al., 2008). Once these sugars are
totally consumed by the fungus, and then begin the secondary metabolism, which consists of the breakdown, decomposition and mineralisation of structural carbohydrates and lignin from substrates by the extracellular enzymes like cellulase, xylanase, lignin peroxidase, manganese peroxidase and laccase (Moyson and Verachtert, 1991; Karunananda et al., 1995; Cohen et al., 2002).

The lignolytic system of *Pleurotus* spp. has been extensively studied in recent years by fungal cultivation on different substrates. During cultivation on lignocellulosic substrates enzyme activities change when shift between substrate colonization and fructification stages of mushroom growth (Oziel et al., 2008).

As in *Agaricus* spp, laccase and endoglucanase activities have been associated with the colonization and fructification stages of the *Pleurotus* mushroom, observing that the spawn running on the substrates is a critical period for the cultures. On the other hand, the laccase has shown to act not only in the biodegradation of lignin, but also in the detoxification of the substrate and defence from antagonistic moulds. Therefore the strains with high production of this enzyme could have advantages during the colonization and biodegradation of the substrate by the mushroom (Cohen et al., 2002).

### 2.7.1 Factors affecting extracellular enzyme production during growth of *Pleurotus* spp. on lignocellulosic substrates

Substrate is a key component in *Pleurotus* mushroom cultivation. Substrate must be suitable for the growth and fruiting of fungus. But, the utilization of insoluble lignocellulosic substrates by edible *Pleurotus* mushrooms is dependent upon the production and secretion by these fungi of enzymes (cellulases, hemicellulases, and ligninases) that bring about hydrolysis/oxidation of the macromolecular
cellulose, hemicellulose and lignin components, respectively, thereby liberating low molecular growth nutrients (Buswell et al., 1996).

Production of these enzymes by the fungal mycelium is a crucial part of the colonization process and an important determinant of mushroom yields. Decay rate of plant debris is proportional to its lignin content. Lignin degradation appears to be associated with the vegetative phase of fungal growth, while cellulose degradation is associated with fruit-body formation. Several studies show that substrate composition does influence enzymatic activity (Karunanandaa et al., 1995). Lignin content of the substrate affects cellulase activity and consequently cellulose utilization. *Pleurotus* spp. grown on lignin-rich substrates show more laccase activity much greater than the activity of cellulases and hemicellulases. Climate is another factor for successful mushroom cultivation. Indoor cultivations are done for precise climate control. But it is very costly.

2.7.2 Factors affecting extracellular enzyme production during growth of *Pleurotus* spp. in solid state fermentation systems

Various lignocellulosic substrates and white-rot fungi have been used successfully in submerged and solid-state fermentation for lignocellulolytic enzyme production. The data obtained process that the type and composition of lignocellulosic substrate appear to determine the type and amount of enzyme produced by basidiomycetes (Elisashvili et al., 2006). Compared with submerged fermentation, solid-state fermentation provides certain advantages of fungal enzyme production with the aspect of application in bioprocesses such as biobleaching, biopulping, bioremediation etc (Stajic et al., 2006). Moreover, it has been shown that, during solid-state fermentation of lignocellulosic materials, some fungi produce a different set of enzymes compared with synthetic liquid cultures.
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The type of fungal strain and cultural conditions can significantly affect the content of different cell components, which in turn may influence the fungal growth and metabolite production in a fermentation process. In a fermentation process, the measurement of microbial biomass is often necessary since metabolic activity is strongly related to the growth rate and the actual biomass present. Owing to the difficulty in estimating the mycelial growth in SSF involving fungi, an indirect method of measurement was employed. Since *P. ostreatus* is a potent producer of cellulase, the cellulolytic activity was determined along with total protein yield. Sugars in general facilitate the growth and proliferation of saprophytic fungi and thus result in an increase in biomass (Erkel, 1992).

The other factors important for SSF are moisture content, carbon, and nitrogen sources in the fermentation media. Cellulosic wastes themselves acted as the carbon source, so no additional carbon source was required in the fermentation medium. L-asparagine was added as a source of organic nitrogen. The whole fermentation medium worked as a complex medium for growth of fermenting fungus. To put biological delignification processes into practice, it is essential to maximize both the rate and the specificity of lignin breakdown by providing conditions that favor lignin degradation and discourage carbohydrate consumption (Stajic et al., 2006).

Moisture in the culture medium greatly affects the mycelial growth rate and metabolic activity. In case of solid state fermentation with filamentous fungi, the available moisture in the substrate provides much needed turgor pressure which enables better penetration of hyphal tip into solid substrate. The yields of cellulase and protein progressively increased with an increase in initial moisture ratio, probably because availability of adequate moisture content initiated rapid uptake of water leading to hyphal cell elongation and elaborate mycelial run within the saturated straw fibres. The good water holding capacity of rice straw also
facilitated better mycelial development (Edwards et al., 2011). This observation may be further explained by a previous study on microbial biomass and its activity in birch litter which reported a strong correlation between moisture content and respiration. An initial increase in the respiration rate was recorded in the continuously wet samples which became relatively constant thereafter (Karunanandaa et al., 1995).

Effect of different nitrogen sources on ligninolytic enzyme production by *Pleurotus* spp. have been studied during solid-state fermentation of grapevine sawdust, showing a promising potential in biotechnological applications. Organic nitrogen sources have been shown to stimulate enzyme production more than inorganic sources. Also, trace elements that can interact with the enzymes or participate in gene regulation processes are necessary for synthesis and function of the ligninolytic enzymes, while their higher amounts present potential inhibitors of enzymatic reactions (Cohen et al., 2002).

The nature of the substrate as well as the cultivation method affects the expression of lignocellulolytic enzymes. The study conducted by Elisashvili et al. revealed that SSF of tree leaves by *Pleurotus* spp. was favourable for laccase and manganese peroxidase (MnP) production. Furthermore, co-culturing can be an effective method for biopulping and improvement of lignin degradation. It was found that co-culturing *P. ostreatus* with *Ceriporiopsis subvermispora* significantly stimulated lignin degradation when compared to monocultures. Laccase production and MnP activity were stimulated in co-cultures of *P. ostreatus* with *C. subvermispora* or *Physisporinus rivulosus* and a change in the isoform composition of those enzymes was also observed (Elisashvili et al., 2006).

Banana leaf waste was a better substrate than banana pseudostem waste in the production of extracellular enzymes by *P. ostreatus* and *P. sajor-caju* in SSF and
is a potential alternative to other agro-waste substrates. The yields were, however, too low and commercially not viable. It was suggested that a larger surface area of banana leaf waste could be a determining factor for better enzyme production. This is in agreement with Zhang et al. who reported that \textit{P. sajor-caju} grew faster and provided better yields on ground straw than on chopped straw. There is, however, a substrate particle size limit, as more finely ground straw inhibited growth. Optimal particle sizes should therefore be determined for all applications (Zhang et al., 2002).

Stajic et al. reported the maximum activity of lignocellulose degrading enzymes at 16-24 days of solid state fermentation, in 2006. They used banana pseudostem as lignocellulosic biomass. Cohen et al. (2002) found the maximum enzyme activity at 10 days in pseudostem and at 20 days on leaf biomass fermentation in both \textit{P. ostreatus} and \textit{P. sajor-caju}. They also reported the highest laccase enzyme activity at 10 days and carboxy-methyl cellulase activity at 5-8 days on solid state fermentation of saw dust and straw in \textit{P. ostreatus} and \textit{P. sajor-caju}. Therefore, fewer days required for maximum enzyme activities, and the increased sugar and protein production on saw dust, straw, and bagasse, compared to banana leaves or pseudostems may be due to the effect of NaOH pretreatment, or lignocellulolytic enzyme activities being higher on these substrates (Berlin et al., 2006).

\textbf{2.8 Major extracellular enzymes of \textit{Pleurotus} spp.}

\textbf{2.8.1 Cellulase}

\textit{Pleurotus} spp. can synthesize and release appreciable amounts of cellulolytic enzymes. Fungal cellulases are multi-enzyme complexes. There is a complete set of hydrolytic enzymes involved in the biodegradation of cellulose. It is composed of three main components; exo-\(\beta\)-1,4-glucanase, endo- \(\beta\)-1,4-glucanase, and \(\beta\)-
glucosidase. The enzymatic degradation of cellulose is a complex process. The above said components have been shown to act synergistically in the hydrolysis of cellulose. During degradation, Exo-1, 4-β-glucanase, splits of either cellobiose or glucose from the non-reducing end of cellulose chains, Endo-1, 4-β-glucanase randomly attacks and splits glycosidic linkages over the length of cellulose chain, and β-glucosidase hydrolyse cellobiose and other water soluble cellodextrins to glucose (Oziel et al., 2008).

2.8.2 Hemicellulase

Xylan, the major component of hemicellulose in plant cell walls, is the second most abundant polysaccharide after cellulose. Xylan polymer consists of a main chain of β-1,4- linked D-xylose residues or some substitutes including arabinose, galactose, mannose etc. The complete degradation of this complex structure depends on different enzymes acting in synergism (Buswell et al., 1996). Endo-β-1,4-xylanases hydrolyze β-1,4-bonds between D-xylose residues in the main chain producing xylo-oligosaccharides, and β-Dxylosidases convert xylo-oligosaccharides to xylose monomers.

Some other specific enzymes such as α-Larabnofuranosidase, α-glucuronidase, as well as several esterases have also a cooperative function into the complete degradation of xylan (Karunananda et al., 1995). Xylan is degraded by fungi through the production of a full complement of enzymes. The genera *Trichoderma, Aspergillus, Fusarium* and *Pichia* are considered great producers of xylanases and basidiomycetes usually secret large amount of enzymes to degrade lignocellulosic materials: white-rot fungus *Pleurotus ostreatus*, *Pleurotus sajor-caju* and *Pleurotus florida* are also producer of xylanase enzymes.
The white-rot fungi basidiomycetes have been studied nowadays as xylanase sources, once its extracellular system is produced to act in a wide range of lignocellulosic materials, and because they are considered edible, these mushrooms is highly nutritional and safe being source of important metabolites of interest to the pharmaceutical, cosmetic and food industries (Cohen et al., 2002).

2.8.3 Pectinase

Pectinotytic enzymes or pectinases are a heterogeneous group of related enzymes that hydrolyze the pectic substances. In relation to their activity, polygalacturonase (PG) cleaving á- (1, 4) glycosidic bonds of non-esterified residues and is classified as endo-PG (E.C.3.2.1.15) and exo-PG (E.C.3.2.1.67). A few reports were recorded about the production of endo and exo-polygalacturonase from a white rot fungi. In most industrial applications, fungal PGS prove to be the most useful because of higher enzyme activity and optimum activity at a lower pH range, suited to most fruit and vegetable processing applications (Rashad et al., 2009).

Recently there has been increased interest in the production of microbial polygalacturonase from food processing wastes. For industrial use, polygalacturonase can be produced from several agricultural pectin containing wastes such as apple pomace, but the main source remains citrus peel, lemon peel, coffee pulp and sugar cane bagasse. Rashad et al. studied the preparation and optimization of Pleurotus ostreatus medium using lemon peel as a food processing waste.

2.8.4 Ligninases

*Pleurotus* fungi are known to employ a variety of extracellular oxidative enzymes
to cleave lignin. The main enzymes of fungi taking part in lignin degradation are phenoloxidases such as lignin peroxidase and laccase. Lignin Peroxidase oxidises non-phenolic lignin substructure which leads to extensive degradation of lignin model compounds (Kirk and Farrell, 1987).

Laccases are extracellular phenol oxidases that catalyse the oxidative degradation of aromatic compounds in lignin, while reducing oxygen to water. They are relatively non-specific enzymes. Laccase isoforms vary between species and within species (Oziel et al., 2008). The broad substrate specificity of laccases permits their use in multiple biotechnological and industrial applications as inexpensive biologically and environmentally friendly tools for the pretreatment of lignocellulose for bioethanol production, pulp bleaching, dye degradation, and xenobiotic transformation and detoxification.

2.9 Spent mushroom substrate

After mushroom crops are harvested, millions of tonnes of spent (used) mushroom substrate (SMS) becomes available for other uses (Rinker, 2002). Mushroom industry needs to dispose off more than 50 million tons of used mushroom substrate each year (Fox and Chorover, 1999). According to Rinker (2002), more than 10 million tons of spent Agaricus bisporus substrates are annually produced in the world. Hence, nearly 40% of the spent material is from Agaricus spp..

Mushroom substrate is considered ‘spent substrate’ when one full crop of mushroom has been taken and further extension becomes unremunerative (Wuest and Fahy, 1991). But, in actual case, the used growing medium is far from spent. Recently, the term spent compost or spent mushroom substrate has been replaced by a more appropriate term, ‘post mushroom substrate’ because it is not ‘spent’ and is ready to be further attacked by a new set of microorganisms (Ahlawat et al., 2009).
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The bacterial population in spent mushroom substrate ranges between lowest of $14.33 \times 10^7$ in *Volvariella volvacea* (paddy straw mushroom) to highest of $59.33 \times 10^7$ in oyster mushroom spent substrate. Spent substrate from *P. florida* (oyster mushroom) harbours 5 to 23 fold higher fungal population than other spent substrates (Ahlawat et al., 2009). Among different fungi, *Trichoderma* spp. followed by *Aspergillus* spp. and *Mucor* spp. dominate in different spent substrates (Rinker, 2002). Hence, spent substrate needs heat treatment before being removed from the growing chamber (Rinker, 2002).

Various treatments adds to the cost of cultivation, under normal circumstances, mushroom growers throw away the contaminated SMS far from the farm, without considering environmental repercussion (Rinker, 2002). Without proper treatment, contaminated SMS can cause re-contamination to the mushrooms in the mushroom house. Also, improper disposal of SMS can pose a problem to the environment (Lopez et al., 2008). Conversely, recycling the SMS can increase sustainability and also help farm economy (Rinker, 2002).

During recent years, environmental legislations have forced the mushroom growers to think about better ways of SMS disposal (Ahlawat et al., 2009). The mushroom industry has been considering problems with SMS from an environmental standpoint concerning its effective disposal and recycling (Oh et al., 2000). At the same time the demand for organic residues and compost has also increased several folds considering the ill effects of synthetic pesticides and fertilizers (Ahlawat et al., 2009).

2.10 Weathering of spent mushroom substrate and its environmental impact

Spent mushroom substrate contains high amount of salts and other elements which are harmful to plant growth. Hence, in order to make the spent substrate suitable for agriculture and other purposes, it is exposed to natural climatic conditions like
varied temperature and rainfall, which is called as weathering (Rinker, 2002). Also, large dumped piles of spent mushroom substrate become anaerobic and give off offensive odour and affects air quality (Beyer et al., 1996). Hence, it is often spread onto land and allowed to weather for several years.

The impact of storage and leaching has been explored (Rinker, 2002). The volume of spent substrate decreases over the time (Ahlawat et al., 2009). Weathering causes a slow decrease in the organic matter contents (volatile solids), and makes required improvement in the characteristics of spent substrate because of on-going microbial activity, but at the same time it also releases leachate containing salts and nitrates and other nutrients (Beyer et al., 1996; Rinker, 2002). The run-off from such piles contaminates nearby water sources and pollutes them (Beyer et al., 1996).

There are contradictory reports regarding the pH of fresh and weathered spent substrate. According to Wuest and Fahy (1991), spent substrate has an initial pH of around 7.28, which increases during weathering, while, Chong et al. (1988) found a decrease in pH from its initial value 7.9 to 7.0 on weathering. Devonald (1987) reported pH of the fresh spent substrate in the range of 7.01 and 8.04.

2.11 Uses of spent mushroom substrate

Many beneficial uses for spent mushroom substrate are currently being implemented or evaluated internationally (Rinker, 2002). Spent substrate from *Agaricus bisporus* production is already in wide use as follows: in horticulture as a component of potting soil mixes or as a fertilizer; in agriculture lands to enrich soil; as a casing material in the cultivation of subsequent *Agaricus* crops, in vermiculture as a growing medium, in wetlands for remediation of contaminated water, in the bio-remediation of contaminated soils and as an animal feed (Rinker, 2002; Kim et al., 2011).
Spent substrate from other mushroom species has found acceptance as food for animals, as fuel, and as a matrix for bio-remediation (Rinker, 2002). During cultivation of mushrooms on fresh substrate, there is a gradual depletion of substrate nutrients due to the subsequent utilization by mushroom mycelium. Hence, the substrate which is already ‘spent’ does not support good yield when re-spawned over it (Siddhant, 2009). Good growth and better yield of mushroom can be achieved when spent substrates are supplemented with starch, peptone and wheat bran before re-spawning (Sharma and Jandaik, 1989).

Sharma and Jandaik (1989) also reported that the recycling of Pleurotus waste, supplemented with starch, peptone and wheat bran, for the cultivation of Pleurotus sajor-caju showed a significant yield of mushrooms. Nakaya et al., (2000) recycled Pleurotus cornucopiae waste for the cultivation of two oyster species viz., P. cornucopiae and P. ostreatus. Production of second crop of mushroom from the spent substrate can prove more efficient utilization of the substrate ingredients and can also ameliorate the problem of solid waste disposal in the mushroom industry (Fahy and Wuest, 1984).

Studies have revealed that the spent substrate is rich in organic matter helps in neutralizing acidic soils, adds nutrients to the soils and facilitates plant growth in barren areas (Rinker, 2002). Spent mushroom substrate improves soil health by improving the texture, water holding capacity and nutrient status (Kaddous and Morgans, 1986; Maher, 1991; Beyer et al., 1996). Incorporation of spent substrate in soil leads to an increase in pH as well as the organic carbon content (Kaddous and Morgans, 1986). Shukry et al. (1999) reported that addition of straw in the soil caused an increase in the number of total bacteria, actinomycetes and fungi of the rhizosphere.

The spent mushroom substrate has been found to be a good nutrient source for agriculture, mainly because of its rich nutrient status, high cation exchange
capacity and slow mineralization rate which retain its quality as an organic matter (Ahlawat et al., 2009). During growth on straw substrate, *Pleurotus* releases humic acids like fractions, which when added to the soil, increase its fertility, thus making the soil suitable for raising vegetables (Zadrazil and Brunnert, 1980; Kaddous and Morgans, 1986).

Spent *Pleurotus* substrate contains high percentage of three primary nutrients – nitrogen (N), phosphorus (P) and potassium (K), for use as a fertilizer (Rinker, 2002). Spent mushroom substrate normally contains 1.9 : 0.4 : 2.4 %, N – P - K before weathering, and 1.9 : 0.6 : 1.0 %, N – P - K after weathering for 8 - 16 months (Gupta, 2004). The phosphorus and potassium requirements of the crop plants can be fully met by incorporating 5 % of spent substrate by volume, while nitrogen requirement by 25 % of spent substrate by volume (Maher, 1991).

Spent substrate is a nutrient-rich organic by-product of the mushroom industry (Adamovic et al., 1998). Several studies have shown the feasibility of using mushroom waste to produce animal feed (Bae et al., 2006). The *Pleurotus* spp. has capability of converting lignocellulosic material into more digestible protein rich cattle feed (Zadrazil and Kurtzman, 1981). Dietary use of spent mushroom substrate in animal feed could also be feasible from an economic point of view (Oh et al., 2000). Kim et al. (2011) suggested spent substrate as an appropriate forage source for ruminants, due to high levels of fiber. Langar et al. (1982) reported that spent *Agaricus bisporus* substrates could be used as sources of minerals for animals, as they are rich in major and trace minerals. However, Bakshi et al. (1985) reported that spent *Agaricus bisporus* substrates had limited use as animal feed due to their high crude ash content (380 - 530 g / kg).

Spent mushroom substrate originated from different edible mushrooms possesses unique physicochemical and biological properties, which make it an ideal bioremediative agent for various environmental protection activities (Ahlawat et
al., 2009). Spent substrate harbours diverse category of microbes, which have the capability to adsorb organic xenobiotic compounds and other inorganic pollutants and to biologically break it down to harmless substances (Ahlawat and Singh, 2007).

Spent substrate can be mixed with different materials for removal of H$_2$S (Shojaosadati and Siamak, 1999) or volatile organic compounds (Mohseni et al., 1998) from air. There are reports for the use of spent substrate of *Agaricus* spp. in the treatment of metal-contaminated water from coal mines (Anon, 1997; Dvorak et al., 1992; Stark et al., 1994), biological treatment of sewage (International Organic Solutions Corp. 1996) and treatment of waters polluted with radioactive elements and heavy metals (Groudev et al., 1999). Acid mine drainage can be treated using the spent substrate of *Agaricus* spp. and *Lentinula edodes* (Chang, 1999). Spent substrate of *Pleurotus* spp. and *Lentinula edodes* can be used in the reduction of phenol content and toxicity in olive mill waste (Martirani et al., 1996; D’Annibale et al., 1998).

Studies have proved that spent mushroom substrate helps in the bioremediation to reclaim contaminated soils / industrial sites (Rinker, 2002; Ahlawat et al., 2009). The microbes, especially actinomycetes (*Streptomyces* spp. and *Thermomonospora* spp.) present in spent mushroom substrate have strong pollutants catabolising capabilities which result in decreased level of pollutants in contaminated soil after incubation with SMS (Ahlawat and Singh, 2007). The spent mushroom substrate also has the decontamination potential for land sites used for disposal of hazardous wastes (Buswell et al., 1996). Fermor et al. (2000) reported the significant potential of spent mushroom substrate in remediation of lands contaminated with xenobiotic pollutants like pentachlorophenols (PCP), polycyclic aromatic hydrocarbons (PAHS) and aromatic monomers.

Spent substrate of *Agaricus bisporus* can reduce zinc toxicity, and the distribution
of cadmium and lead (Shuman, 1999) among soil fractions. It can degrade polycyclic aromatic hydrocarbons or aromatic monomers (Semple et al., 1998, Fermor et al., 2000, Staments, 2001), inhibit nitrification (Bazin et al., 1991); treat hazardous wastes (Buswell et al., 1996); and stabilise disturbed commercial sites (Rupert, 1995). Spent substrate of Pleurotus spp. can degrade polycyclic aromatic hydrocarbons in age-creosote contaminated soil (Eggen, 1999). Spent substrate of Lentinula edodes, Pleurotus spp. and Agaricus bisporus can degrade pentachlorophenol (Chiu et al., 2000; Semple et al., 1998).

The use of hydrolytic and oxidative enzymes from Pleurotus spp. is one of the most important aspects for the biodegradation of organo-pollutants, xenobiotics and industrial contaminants (Cohen et al., 2002). The spent substrate from oyster mushroom shows high activity of laccase, manganese peroxidase and arylalcohol oxidase in comparison of spent substrate of button and paddy straw mushrooms (Ahlawat et al., 2009). Button mushroom spent substrate shows higher activity of lignin peroxidase only (Ahlawat et al., 2004). These enzymes can be used for various biotechnological and environmental applications. Lignocellulolytic enzymes have significant potential applications in the chemical, fuel, textile, agriculture and pulp and paper industries (Elisashvili et al., 2006).

2.12 Applications of spent mushroom substrate

2.12.1 Source of plant fibre

Plant fibres are long, stretched, thick-walled cells and are mainly made up of cellulose. Vegetable fibres are fibres produced from bast, seed, leaf and sheath of plants. They are discrete single entities as in cotton; lignocellulosic meshy as in jute; long as in pineapple leaf; or short as in areca nut. Some of them are strong and fine with high length to breadth aspect ratio for good spinnability into yarn for fabric (Das and Mukherjee, 2008). Primarily, cotton is used for apparel; jute for packaging; ramie for currency paper blanks; sisal for rope; sun hemp for tissue
paper etc. Ramie is the strongest amongst all the vegetable fibres and therefore, it has specialised applications (Das and Mukherjee, 2008). Unlike plastics, vegetable fibres are biodegradable, annually renewable, non-carcinogenic and therefore health-friendly.

Several industrial sectors, such as the paper, textile and composite industries are making persistent and ever increasing demands for cellulose fiber (Focher et al., 2001). Also, lignocellulosic raw material is in abundance. Common methods to extract fibres from plant material are fairly straight forward processes, though long, smelly and labour intensive. The basic principle is to encourage the softer parts (non-cellulosic) of the plant to rot, so that only the stronger cellulose fibres remain. Cellulose and hemicellulose components of the plant cell walls are intimately associated with lignin moiety. The individual cellulose fibres are bonded together with a lignin-rich region known as middle lamella. Hemicelluloses (mainly xylan) are also intimately associated with the cellulose fibrils, embedding the cellulose in a matrix (Olesen, 1997).

Fibres are separated from each other by the dissociation of lignin, pectin and xylan from the middle lamella and primary wall of plant material. White-rots of Pleurotus sp. can produce extracellular enzymes to degrade all the major components of plant material, at the same time (Eriksson et al., 1990). However, these enzymes are secreted in different proportions. They remove lignin preferentially, with limited degradation to cellulose. Hence, spent substrate of Pleurotus sp. is reported to be a good source of fibre.

2.12.2 Source of ethanol

Ethanol is an oxygenated fuel with high octane value like that of petroleum fuels (Bishnu et al., 2011). It is known to run combustion engines at higher compression ratios and thus provides superior performance (Wheals et. al., 1999). The world
population is estimated to increase from 6.7 billion to 8 billion by 2030 (USCB, 2008). On the other hand, global oil production is expected to decline from 25 billion barrels to 5 billion barrels by 2050 (Campbell and Laherree, 1998; Kumar et al., 2011). Demand for transportation fuels across the globe is increasing. This demand is abnormally affecting developing countries in particular.

Countries that totally depend on the import of fossil fuels cannot ignore the potential of bioethanol derived from lignocellulosic biomass (Bishnu et al., 2011). The blending of ethanol into petroleum-based automobile fuels can significantly decrease petroleum use and release of greenhouse gas emissions to an extent of 85% (Perlack et al., 2005; Bishnu et al., 2011). Further, ethanol can be a safer alternative to the common additive, methyl tertiary butyl ether (MTBE), in gasoline. MTBE is toxic and is a known contaminant in ground water (McCarthy and Tiemann, 1998; Wang et al., 1999). The US Environmental Protection Agency recently announced the beginning of regulatory action to eliminate MTBE in gasoline (Browner, 2000). Owing to depleting reserves and competing industrial needs of petrochemical feedstocks, there is global emphasis in ethanol production by microbial fermentation process (Kumar et al., 2011).

Ethanol may be produced either from petroleum products, or from biomass through fermentation. Currently, most of the ethanol produced from renewable biomass resources comes from sugarcane and starchy grains. This is partly due to ease of substrate handling and processing. On the other hand, use of sugarcane and food grains to produce bio-ethanol has caused significant stress on food prices and food security. The long-term viability of this process is in question because it will require significantly increased amounts of cultivatable land and significant hike in food prices that will ultimately lead to food insecurity (Mitchell, 2008). Accordingly, the recent focus has been on lignocellulosic materials as a source for bio-ethanol.
Lignocellulosic materials are in abundance and they are renewable (Bishnu et al., 2011). Hence, bioethanol production from lignocellulosic biomass holds tremendous potential in terms of meeting energy needs, and providing environmental benefits (Bishnu et al., 2011). Significant efforts are being made to produce ethanol from lignocellulosic biomass (almost 50% of all biomass in the biosphere) such as agriculture residues (Bothast and Saha, 1997). The technological advances in recent years are promising to produce ethanol at low cost from lignocellulosic biomass. Ethanol production generally involves hydrolysis of lignocellulosic biomass to fermentable sugars followed by fermentation of such sugars to ethanol (Bishnu et al., 2011).

The improvements in pretreatment processes, improvement in efficacy of enzymatic hydrolysis, development of efficient fermentation processes, efficient technologies to recover ethanol and removal of toxic byproducts will decrease the operating and capital costs (Bishnu et al., 2011). The reduction in cost of ethanol production can be achieved also by reducing the cost of raw material and cofermenting hexose and pentose sugars in the same tanks (Bishnu et al., 2011).

Many countries are moving towards developing or have already developed technologies to exploit the potential of lignocellulosic materials for the production of bioethanol. The leading nations in bioethanol production are Brazil and the USA, and USA is the world’s largest producer of bioethanol (Carere et al., 2008). The US fuel ethanol industry produced more than 6.2 billion litres of ethanol in 2000, most of which was produced from corn (MacDonald et al., 2001). Asian countries altogether account for about 14% of world’s bioethanol production (Bishnu et al., 2011).
2.12.2.1 Pretreatment of lignocellulosic biomass

The first step in the overall process of lignocellulosic fermentation is the pretreatment of biomass. This is the most important and rate limiting step in the overall process. Pre-treatment (i) breaks the lignin-hemicellulose-pectin complex around cellulose, (ii) disrupt / loosen-up the crystalline structure of cellulose and (iii) increase the porosity of the biomass (Bishnu et al., 2011). Biomass pre-treatment technologies can change / remove structural and compositional constraints to improve hydrolysis rate and increase yields of fermentable sugars from cellulose and will have a significant impact on the overall process (Sun and Cheng, 2002; Hamelinck et al., 2010; Mosier et al., 2005; Huber et al., 2006; Yang and Wyman, 2008). An ideal pretreatment process should yield high levels of pentoses and the hydrolysates should not have any inhibitory substances and the process is cost effective (Lynd, 1996). The current methods in practice include mechanical, physical, chemical and biological processes or their combination.

2.12.2.1.1 Mechanical pre-treatment

Waste materials can be pre-treated by a combination of chipping, grinding and milling to reduce cellulose crystallinity (Millet et al., 1976). The power requirement of mechanical pre-treatment of agricultural materials depends on the final particle size and the waste biomass characteristics (Cadoche and Lopez, 1989).

2.12.2.1.2 Physical pretreatment

Hydrothermal processes use water, steam or both, and heat, for biomass pre-treatment (Carvalheiro et al., 2008). Hydrothermal treatments mainly include
liquid hot water (autohydrolysis) and steam explosion between 150 °C and 230 °C (Garrote et al., 1999; Carvalheiro et al., 2008). It was hypothesized that CO\textsubscript{2} would form carbonic acid and increase the hydrolysis rate in lignocellulosics. The yields were relatively low compared to steam or ammonia explosion pretreatment, but high compared to the enzymatic hydrolysis without pretreatment.

Steam explosion is the most commonly used method for pretreatment of lignocellulosic materials. The process causes hemicellulose degradation and lignin transformation due to high temperature, thus increasing the potential of cellulose hydrolysis (McMillan, 1994; Duff and Murray, 1996; Mackie et al., 1985). Because of the formation of degradation products that are inhibitory to microbial growth, enzymatic hydrolysis, and fermentation, pretreated biomass needs to be washed by water to remove the inhibitory materials along with water-soluble hemicellulose (McMillan, 1994). The water wash decreases the overall saccharification yields due to the removal of soluble sugars, such as those generated by hydrolysis of hemicellulose (Mes-Hartree et al., 1988).

In pyrolysis, the materials are treated at temperatures greater than 300 °C and cellulose rapidly decomposes to produce gaseous products and residual char (Kilzer and Broido, 1979; Shafizadeh and Brad-bury, 1979). The decomposition is much slower and less volatile products are formed at lower temperatures (Fan et al., 1987; Shafizadeh et al., 1979). AFEX is another type of physico-chemical pretreatment in which lignocellulosic materials are exposed to liquid ammonia at high temperature and pressure for a period of time, and then the pressure is swiftly reduced. The AFEX pretreatment does not significantly solubilize hemicellulose compared to acid pretreatment and acid-catalyzed steam explosion (Mes-Hartree et al., 1988; Vlasenko et al., 1997; Holtzapple et al., 1992).
2.12.2.1.3 Chemical pretreatment

In these methods, lignocellulosic materials were pre-treated with powerful oxidizing agents such as per-acetic acid or hydrogen peroxide (Gould, 1984). Teixeira and associates observed ethanol yields of 98% when the lignocellulosic biomass was pretreated with 21% peracetic acid (Teixeira et al., 1999). More recent processes have employed treatment with hydrogen peroxide (Saha and Cotta, 2007), sulfite (Kuhad et al., 1999; Azzam, 1989) etc.

In the organosolv process, an organic or aqueous organic solvent mixture with inorganic acid catalysts is used to break the internal lignin and hemicellulose bonds (Chum et al., 1988; Thring et al., 1990). Recovery process has been shown to isolate lignin as a solid and carbohydrates as syrup (Lora and Aziz, 1985; Johansson et al., 1987; Aziz and Sarkanen, 1989). The disadvantages are that the process requires expensive high pressure equipment (Pan et al., 2005; Yamashita et al., 2010; Sarkanen, 1980; Lora and Aziz, 1985; Johansson et al., 1987; Ben-Ghedalia and Miron, 1981). Ozone can be used to degrade lignin and hemicellulose in many lignocellulosic materials such as wheat straw (Ben-Ghedalia and Miron, 1981) and bagasse (Neely, 1984). The degradation was essentially limited to lignin, and hemicellulose was slightly attacked, but cellulose was hardly affected.

2.12.2.1.4 Biological pre-treatment

In biological pretreatment processes, microorganisms such as brown-, white- and soft-rot fungi are used to degrade lignin, hemicellulose and pectin in waste materials (Schurz, 1978; Cardona et al., 2010). Brown rots mainly attack cellulose, while white and soft rots attack both cellulose and lignin. White-rot fungi are the most effective basidiomycetes for biological pretreatment of lignocellulosic
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materials (Fan et al., 1987). One of the readily available and cheap source of hydrolytic and oxidising enzymes for the pre-treatment as well as hydrolysis of lignocellulosic biomass, is the spent substrate of commercially grown edible white-rot (Avneesh et al., 2011).

Spent mushroom substrate is a suitable raw material for ethanol production. Hatakka (1994) studied the pretreatment of wheat straw by 19 white-rot fungi and found that 35 % of the straw was converted to reducing sugars by Pleurotus ostreatus in five weeks. The advantages of biological pre-treatment include low energy requirement and mild environmental conditions. However, the rate of hydrolysis in most biological pretreatment processes is very low.

2.12.2.2 Hydrolysis

Post pretreatment, the recalcitrant lignocellulosic biomass becomes susceptible to acid and/or enzymatic hydrolysis as the cellulosic microfibrils are exposed and/or accessible to hydrolyzing agents (Bishnu et al., 2011). Further steps involve isolation and hydrolysis of cellulose to generate fermentable sugars (saccharification) (Bishnu et al., 2011). This process is mainly accomplished by enzymatic methods using cellulases (Bishnu et al., 2011). Mild acid hydrolysis using sulfuric and hydrochloric acids is an alternative procedure (Bishnu et al., 2011). The goal of this process is to generate fermentable monomeric sugars from hemicellulose and cellulose content of lignocellulosic biomass. This can be accomplished by two different processes, namely, acid hydrolysis and enzymatic hydrolysis.

Although several detoxification methods, such as activated charcoal adsorption and lime treatment process, have been devised, an appropriate strategy for efficient hydrolysis of cellulose to fermentable sugars is still lacking (Kaya et al.,
2000; Aden et al., 2002). Following pretreatment, hydrolysis process is applied to lignocellulosic biomass in a two-step (stage) process because the pentose sugars (first stage hydrolysis) degrade / decompose more rapidly than hexose sugars (second stage hydrolysis). Solid fraction of cellulose and lignin are subjected to second stage hydrolysis (Bishnu et al., 2011).

2.12.2.2.1 Hydrolysis of hemicellulose

The pre-treated biomass is subjected to filtration to separate liquids (hemicellulose hydrolysate) and solid (lignin + cellulose) (Bishnu et al., 2011). In the pretreatment process, small amounts of cellulose and most of hemicellulose is hydrolyzed to sugar monomers; mainly D - xylose and D - arabinose. Hemicellulose is usually treated as a secondary stream due to lack of efficient fermentation of hemicellulosic sugars to ethanol (Florbela et al., 2008). The liquid portion is sent to a xylose (pentose) fermentation column for ethanol production (Bishnu et al., 2011). The pentoses (D - xylose and D - arabinose) from hemicellulose hydrolysis are not easily utilized by saccharomyces strains; therefore, genetically modified strains of Pichia stipitis, Zymomonas mobilis, are used for their fermentation (Bishnu et al., 2011).

2.12.2.2.2 Hydrolysis of cellulose

Acid hydrolysis

Mineral acids such as sulfuric acid, hydrochloric acid, hydrofluoric acid and nitric acid are widely employed for the hydrolysis of lignocellulosic biomass. Among these, the oldest and best understood process utilizes sulphuric acid. Use of hydrochloric acid, although not commonly used, has technical advantages over the sulfuric acid process, given it is relatively volatile and can be recovered by
vacuum stripping methods (Bishnu et al., 2011). The sulfuric acid-based hydrolysis process is operated under two different conditions; (i) a process that uses high sulfuric acid concentration that operates at a lower temperature and, (ii) a process that uses low sulfuric acid concentration and operates at a higher temperature. Among the two, the latter is most commonly used.

Use of concentrated sulfuric acid alone has been shown to (i) yield very high levels of sugar (90 %), (ii) can handle diverse feedstock, (iii) is relatively rapid (10 to 12 hours), and (iv) causes less degradation (Graf and Koehler, 2000; USDOE, 2003; Hamelinck et al., 2010). Concentrated acid-based processes are very expensive and cause significant operational problems (Sun and Cheng, 2002; Galbe and Zacchi, 2002; Hamelinck et al., 2010). Although they are powerful agents for cellulose hydrolysis, concentrated acids are toxic, corrosive and hazardous and require reactors that are resistant to corrosion (Shleser, 1994). In addition, the concentrated acid must be recovered after hydrolysis (Sivers and Zacchi, 1995; Gupta et al., 2009). Acid recovery is a key step for economic viability of concentrated acid pre-treatments, and these neutralization costs have hampered general use of these pre-treatments (Goldstein et al., 1983).

Dilute sulphuric acid processes have been most favored for industrial application, because it achieves reasonably high sugar yields from hemicellulose (Galbe and Zacchi, 2002; Hamelinck et al., 2010). Compared to concentrated acid hydrolysis, this pretreatment generates lower degradation products as well much less corrosion problems in hydrolysis tanks, pipes, etc (Carvalheiro et al., 2008). The dilute sulfuric acid pretreatment can achieve high reaction rates and significantly improve cellulose hydrolysis (Esteghlalian et al., 1997). At moderate temperature, direct saccharification suffers from low yields because of sugar decomposition. But high temperature in dilute acid treatment is favorable for cellulose hydrolysis (McMillan, 1994).
Recently developed dilute acid hydrolysis processes use less severe conditions and achieve high xylan to xylose conversion yields. Achieving high xylan to xylose conversion yields is necessary to achieve favorable overall process economics because xylan accounts for up to a third of the total carbohydrate in many lignocellulosic materials (Hinman et al., 1992). Although dilute acid pretreatment can significantly improve the cellulose hydrolysis, its cost is usually higher than some physico-chemical pretreatment processes such as steam explosion or AFEX. Also, a neutralization of pH is necessary for the downstream enzymatic hydrolysis or fermentation processes. This process produces relatively large number of undesirable byproducts as compared to the concentrated acid process (Bishnu et al., 2011).

**Alkaline hydrolysis**

Some bases can also be used for pretreatment of lignocellulosic materials and the effect of alkaline pretreatment depends on the lignin content of the materials (Fan et al., 1987; McMillan, 1994). Alkali pretreatment increases cellulose digestibility (Carvalheiro et al., 2008). Most commonly used alkali in the alkali pretreatment processes are NaOH and Ca(OH)$_2$. This process results in (i) the removal of all lignin and part of hemicellulose, and (ii) increased reactivity of cellulose in further hydrolysis steps (Hamelinck et al., 2010), especially, enzymatic hydrolysis. Between NaOH and Ca(OH)$_2$, pretreatment with Ca(OH)$_2$ is preferable because it is less expensive, more safer as compared to NaOH and it can be easily recovered from the hydrolysate by reaction with CO$_2$ (Mosier et al., 2005).

The mechanism of alkaline hydrolysis is believed to be saponification of intermolecular ester bonds cross-linking xylan hemicelluloses and other components, for example, lignin and other hemicellulose. The porosity of the
lignocellulosic materials increases with the removal of the cross-links (Tarkow and Feist, 1969). Conversely to acid or hydrothermal processes, alkaline-based methods are more effective for lignin solubilisation exhibiting only minor cellulose and hemicellulose solubilisation, excepting ammonia recycling percolation treatment, which yield biomass solids mostly containing cellulose (Sun and Cheng, 2002; Hamelinck et al., 2010; Wyman et al., 2005).

Pretreatment with NaOH increases the digestibility cellulose from 14 to 55% while decreasing the lignin content from 25 to 20% (Kumar et al., 2011). Alkali pretreatment process shows decreased sugar degradation and is more effective on agriculture residues as compared to wood materials (Kumar et al., 2011). Dilute NaOH pretreatment was also effective for the hydrolysis of straws with relatively low lignin content of 10 - 18% (Bjerre et al., 1996). Dilute NaOH treatment of lignocellulosic materials causes swelling, leading to an increase in internal surface area, a decrease in the degree of polymerization, a decrease in crystallinity, separation of structural linkages between lignin and carbohydrates, and disruption of the lignin structure (Fan et al., 1987; Taherzadeh and Karimi, 2008). However, no effect of dilute NaOH pretreatment was observed for softwoods with lignin content greater than 26% (Millet et al., 1976).

**Enzymatic hydrolysis**

During enzymatic hydrolysis, cellulose is degraded by enzymes known as cellulases that are able to hydrolyse the cellulose polymer to its monomer (Fatma et al., 2010). The factors that affect the enzymatic hydrolysis of cellulose include substrates, cellulase activity, and reaction conditions such as temperature, pH, as well as other parameters (Cantwell et al., 1988; Durand et al., 1988; Orpin, 1988).
Cellulases are usually a mixture of several enzymes. At least three major groups of cellulases are involved in the hydrolysis process: (1) endoglucanase (EG, endo-1,4-D-glucanohydrolase, or EC 3.2.1.4.) which attacks regions of low crystallinity in the cellulose fiber randomly and cleave the cellulose chains to form glucose, cellobiose and cellotriose, creating free chain-ends; (2) exoglucanase or celllobiohydrolase (CBH, 1,4-b-D-glucan cellbiohydrolase, or EC 3.2.1.91.) which attack the non-reducing end of cellulose and degrades the molecule further by removing cellobiose units from the free chain-ends; (3) β-glucosidase (EC 3.2.1.21) or cellobiase which hydrolyzes cellobiose to produce D-glucose (Coughlan and Ljungdahl, 1988).

In addition to the three major groups of cellulase enzymes, there are also a number of ancillary enzymes that attack hemicellulose, such as glucuronidase, acetylesterase, xylanase, b-xylosidase, galactomannanase and glucomannanase (Duff and Murray, 1996). The factors affecting activity of cellulases include enzyme source and the concentration of enzyme. To improve the rate of the enzymatic hydrolysis and the yield of sugar, research has focused on optimizing the hydrolysis process and enhancing cellulase activity (Cantwell et al., 1988; Durand et al., 1988; Orpin, 1988).

Bacteria and fungi produce cellulases that hydrolyze of lignocellulosic materials. These microorganisms can be aerobic or anaerobic and mesophilic or thermophilic. Bacteria belonging to genera of Clostridium, Cellulomonas, Bacillus, Thermomonospora, Erwinia, Acetovibrio, and Streptomyces are known to produce Cellulase (Bisaria and Chandrakant, 1998). Anaerobic bacterial species such as Clostridium phytofermentans, Clostridium thermocellum and Clostridium papyrosolvens produces cellulases with high specific activity (Duff and Murray, 1996; Joshi, 1997; Bisaria and Chandrakant, 1998).
Fungi known to produce cellulases include *Sclerotium rolfsii*, *Phanerochaete chrysosporium* and various species of *Trichoderma, Aspergillus* and *Penicillium* (Sternberg, 1976; Fan et al., 1987; Duff and Murray, 1996). Among the fungi, *Trichoderma* species have been extensively studied for cellulase production (Sternberg, 1976). Most commercial glucanases (cellulases) are produced by *Trichoderma reesei* and β-D-glucosidase is produced from *Aspergillus niger* (Kaur et al., 2007).

Cellulase enzyme loadings in hydrolysis vary from 7 to 33 FPU / g substrate, depending on the type and concentration of substrates. Enzymatic hydrolysis of cellulose consists of three steps: adsorption of cellulase enzymes onto the surface of the cellulose, the biodegradation of cellulose to fermentable sugars, and desorption of cellulase. Cellulase activity decreases during the hydrolysis. The irreversible adsorption of cellulase on cellulose is partially responsible for this deactivation (Converse et al., 1988). Increasing the dosage of cellulases in the process, to a certain extent, can enhance the yield and rate of the hydrolysis, but would significantly increase the cost of the process.

Cellulase dosage of 10 FPU / g cellulose is often used in laboratory studies because it provides a hydrolysis profile with high levels of glucose yield in a reasonable time (48 - 72 h) at a reasonable enzyme cost (Gregg and Saddler, 1996). The yield of fermentable sugar levels obtained from pre-treated biomass increases as the enzyme load increases (Yang and Wyman, 2008). According to Kim and his associate (2005), effective concentration of enzyme for cellulose hydrolysis has been determined to be 10 to 60 FPU (filter paper units) per gram of dry cellulose or glucon- glucanase- β - D - glucosidase ratio of 1 – 75 - 2 IU.

A mixture of hemicellulases or pectinases with cellulases exhibited a significant increase in the extent of cellulose conversion (Ghose and Bisaria, 1979; Beldman
et al., 1984; Berlin et al., 2007). Non-ionic surfactants are believed to be suitable for enhancing the cellulose hydrolysis. The rate of enzymatic hydrolysis was improved by 33 % using Tween 80 as a surfactant in the hydrolysis of newspaper (Castanon and Wilke, 1981). Cellulase activity is inhibited by cellobiose and to a lesser extent by glucose. Several methods have been developed to reduce the inhibition, including the use of high concentrations of enzymes, the supplementation of β - glucosidases during hydrolysis, and the removal of sugars during hydrolysis by ultrafiltration or simultaneous saccharification and solid state fermentation (SSF).

The SSF process has been extensively studied to reduce the inhibition of end products of hydrolysis (Takagi et al., 1977; Blotkamp et al., 1978; Szczodrak and Targonski, 1989; Saxena and Rai, 1992; Philippidis et al., 1993; Zheng et al., 1998). Reducing the cost of cellulase enzyme production is a key issue in the enzymatic hydrolysis of lignocellulosic materials. Cellulases can be recovered from the liquid supernatant or the solid residues. Enzyme recycling can effectively increase the rate and yield of the hydrolysis and lower the enzyme cost (Mes-Hartree et al., 1988).

2.12.2.3 Fermentation

During fermentation, both pentose and hexose sugars are fermented to ethanol under anaerobic/aerobic conditions. Increased yield of ethanol production by microbial fermentation depends on the use of ideal microbial strain, appropriate fermentation substrate and suitable process technology (Brooks, 2008).

Economical production of ethanol from lignocellulose hydrolysates requires high concentrations of fermentable sugars in it. High osmolality of the media prevents the action of ethanologens, as most of these microorganisms used for ethanol
production are incapable of performing at high sugar and salt concentrations (Kurian et al., 2010). Common problems associated with fermentation of sugar are the high temperatures (35 - 45 °C) and high ethanol concentration (over 20 %). Tolerance to high temperatures and ethanol concentrations are important factors of microorganisms for increasing efficiency at industrial scale (Jimenez and Benitez, 1986; Mehdikhani et al., 2011). Industrial production of ethanol from lignocellulosic hydrolysates requires the use of microorganism capable of utilizing the different types of sugars present in it (Balat et al., 2008; Bettiga et al., 2009).

*Saccharomyces cerevisiae* is the most favored organism for ethanol production from hexoses. It is tolerant to temperature and high sugar and ethanol concentrations (Osho, 2005). High ethanol tolerant strains are able to extend the process of fermentation for longer time and produce distinct products in the presence of ethanol (Świeciło et al., 2000). Invertase is one of the important extracellular enzymes in *Saccharomyces* that is responsible for converting sucrose to its subunits, glucose and fructose (Sengupta et al., 2000). Despite its extensive use, it has a number of disadvantages, such as high aeration cost, high biomass production and low temperature and ethanol tolerances (Saigal, 1993). *Zymomonas mobilis* is a potential bacterium for ethanol production (Parmjit et al., 2006). But, despite various efforts undertaken worldwide, *Zymomonas* is not yet ready to compete successfully with the yeast at industrial scale (Parmjit et al., 2006). Yeasts have higher ethanol tolerance than bacteria (Kurian et al., 2010). *P. stipitis* and *Candida shehatae* are capable of fermenting both hexose (glucose) and pentose (xylose) sugars to ethanol (Parekh and Wyman, 1986). Genetically engineered strains of *Escherichia coli*, *S. cerevisiae*, and *Z. mobilis* have been developed to ferment xylose (Kim and Holtzapple, 2005).

An optimal process for fermentation uses a broth containing *Saccharomyces cerevisiae* supplemented with 22 % (w/v) sugar, 1 % (w/v) of each of ammonium sulphate and potassium dihydrogen phosphate, and fermented at pH 5.0 and 30 °C.
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(Junior et al., 2009). Under such conditions a typical strain of \textit{S. cerevisiae} is capable of producing 46.1 g ethanol / L broth (Maziar, 2010). Molasses medium is the most commercially used medium for ethanol production (Parmjit et al., 2006).

2.12.3 Bioremediation of phenol

 Phenol is an aromatic hydrocarbon which exists as a colorless or white solid in its pure state. It is a toxic organic compound which is constantly introduced into our environment and found to affect aquatic life even at relatively low concentration (5 - 25 mg / L), causing ecological imbalance. It is found in the water effluent of industries like coke oven units, oil refineries, plastics, leather and paint industries and paper and pulp industries (Haghseresht and Lu, 1998). It has high bioaccumulation rate along the food chain. Phenol can be absorbed by our body through the respiratory organ, skin and alimentary canal. It can restrain the central nervous system and interact with the liver and nephridium (Huang et al., 2008).

Over the past several decades, there is growing concern about widespread contamination of surface and ground water by phenol, due to rapid development of chemical and petrochemical industries. In 1985, WHO imposed a stringent effluent discharge limit of 0.2 mg / L (Mishra and Bhattacharya, 2006). Thereafter, new and tighter regulations coupled with increased enforcement concerning wastewater discharges have been forced in many countries (Khalil et al., 2009). Thus its removal is essential for environmentally sustainable existence.

Conventional treatments to remove phenol from water include solvent extraction, microbial degradation, adsorption on activated carbon and chemical oxidation. These methods although effective and useful, suffer from serious drawbacks such as high costs, low phenol removal efficiency and formation of hazardous by-products. Enzymes are known to be highly selective catalysts. They have shown enormous biotechnological potential as they can be used at a wide level for degradation (Ren and Buschle-Diller, 2007) for removing targeted substances
from wastewaters and detoxification of agro-industrial residuals with high phenolic contents (Mata, 2005).

It has been hypothesized that the addition of LiP or Laccase enzyme can reduce the concentration of phenolic compounds in wastewater. The white rot fungi, including *Pleurotus* species, have been reported on several occasions as good producers of extracellular ligninolytic enzymes and as active strains for textile dye decolorisation and other pollutants (Fu and Viraraghavan, 2001; Yonni et al., 2004; Nilsson et al., 2006; Zhao et al., 2008). It was also reported that extracellular ligninases such as Lignin Peroxidase (LiP) and Laccase produced by certain *Pleurotus* sp. can degrade phenol (Kirk and Farrell, 1987). One of the readily available and cheap source of enzymes is the spent substrate of commercially grown edible white-rot fungi (Avneesh et al., 2011). Several studies have shown the potential use of the spent substrate of mushrooms in purification of water and soil.