CHAPTER TWO

LITERATURE REVIEW

LIGNOCELLULOSE

The term 'lignocellulose' implies materials that are comprised of mainly cellulose, hemicellulose and lignin. Lignocellulose, has attracted widespread attention as an interesting and renewable raw carbon energy source for many biotechnological processes. It is product, by-product or waste product from both the agricultural and forest product industries and as such, it represents a predominant renewable resource. Although its composition varies with the source, lignin, cellulose and hemicellulose are always present as the major components (Hamer and Egli, 1991).

Lignin

Lignin is a highly recalcitrant compound, forming the second most abundant renewable biopolymer in nature after cellulose (Pointing *et al.*, 2000). It is the major component of wood forming up to 30% of woody plant tissues, too (Kirk and Farrell, 1987). This complex polymer is composed of phenylpropane units interconnected by a variety of carbon-carbon bonds and other ether linkages (Muralidhara *et al.*, 1987).

Biosynthetically, lignin arises from three alcohols: *p*-hydroxycinnamyl (coumaryl) alcohol, which gives rise to *p*-hydroxyphenyl units in the polymer; 4-

hydroxy-3-methoxycinnamyl (coneferyl) alcohol, the guaicyl units; and 3,5-dimethoxy-4-hydroxcinnamyl (sinapyl) alcohol, the syringyl units. Free radical copolymerization of these alcohols produces the heterogeneous, optically inactive, cross-linked, and highly polydisperse polymer (Kirk and Farrell, 1987).

According to Joseph *et al.*, (1994) lignin, a complex 3-dimensional polyaromatic matrix, forms a seal around cellulose microfibrils and exhibits limited covalent association with hemicellulose. It is deposited during maturation of cell walls and some carbohydrates become cross-linked to it chemically and physically. It differs from cellulose both with respect to its chemical composition and its structure, consisting xylans, mannans, arabinans and galactans as the major heteropolymers (Hamer and Egli, 1991; Bastawde, 1992).

Lignocellulosic utilization

The main lignocellulosic products are sawn timber, wood chips for pulping and wood for fuel. For biotechnological processing the most widely available feedstocks, in this category are either the by-products and waste products of sawn timber and wood chips or those of agricultural origin (Hamer and Egli, 1991).

At the present time, very large quantities of lignocellulosic waste are subjected either to usual decay or to wasteful combustion. Processes that make use of lignocellulosic wastes include sensible combustion (e.g. baggase by the cane sugar industry), pulping (e.g. waste paper and a small fraction of the cereal straw produced) and reconstitution (e.g. saw dust and waste chips conversion into artificial board) (Hamer and Egli, 1991).

Considerable research has been undertaken on the thermochemical conversion of lignocellulosic matter (pyrolysis, gastification and catalytic liquefaction) but has yet to make significant impact. Biological technologies that have been proposed for the utilization of lignocellulosic waste include composting, methane generation by digestion in either anaerobic bioreactors or in appropriately constructed landfills and, in specific cases, either utilization as ruminant feeds or for single-cell protein (SCP) production. Research has been directed to saccharification which involves the production of fermentable sugars from lignocellolosic materials to yield either glucose syrup for direct consumption as food-grade product or as a fermention feedstock stream for bulk chemicals or fuel ethanol production (Hamer and Egli, 1991). Fig 2.1 shows the flow diagram of products derived from lignocellulosic materials.

MICROBIAL UTILIZATION OF LIGNOCELLULOSICS

The lignin biodegradation process has an important role in the carbon cycle of the biosphere. Although it can be transformed by a combination of biological and chemical factors the study of this natural process has developed mainly with the use of basidiomycetes. This has been a logical approach since most of the microorganisms involved in lignin biodegradation belong to this class of fungi (Rodriguez *et al.*, 1997). The ultimate natural transformation of lignin (i.e., its complete oxidation to CO_2) is primarily released by white-rot basidiomycetes and particularly their enzymatic system (Morais *et al.*, 1996).

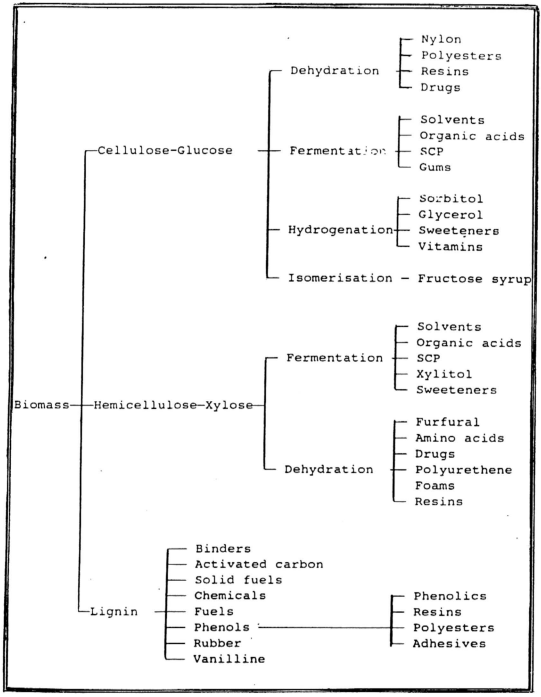

Fig 2.1: Useful products derived from lignocellulosic materials

Source: Ling, (1994)

White-rot fungi are probably the most efficient terrestrial microorganisms capable of utilizing all the polymers of lignocellulosic residues (Wood *et al.*, 1988).

There are many reasons why fungi seem to be the choice organisms for bioconversion although many other organisms have been used as well:

1. Many filamentous fungi are rather fast-growing
2. Filamentous fungi have greater penetrating power into insoluble substrates and are therefore more suited for solid-state fermentation of lignocellulosic materials
3. Fungi have a wide range of enzyme systems capable of utilizing complex mixtures of organic compounds that occur in most wastes.
4. The biomass produced by filamentous fungi can be used without further processing because it provides carbohydrates, lipids, minerals and vitamins as well as protein.
5. Protein content of fungi is high, 35-50% and they have comparably less nucleic acids than single celled organisms.
6. Most filamentous fungi have a faint mushroom-like odour and taste which may be readily acceptable
7. Digestibility and net protein utilization (NPU) without any pretreatment is higher than for single-celled organisms.
8. Filamentous fungi have a greater retention time in the digestive systems

and rich in fiber. According to Nagra *et al.* (1999) fermentation of Guar meal (*Cyamopsis tetragonaloba*) with *Aspergillus niger* and *Fusarium sp.* has potential for use as protein supplement for chickens.

Some research has been undertaken to biologically delignify herbaceous plants to improve the utilization of lignocellulosics by ruminants. Zadrazil (1985) evaluated 235 strains of fungi for their ability to delignify wheat straw and reported extreme variations in activity. Two other white rot fungi selectively degraded hemicellulosic sugars and aromatic constituents, caused low dry-weight losses, and improved *in vitro* digestibility by 63 and 94% (Agosin and Odier, 1985). Similarly, Karunanandaa *et al.* (1992) reported increases and decreases in *in vitro* digestibilities of corn and rice straw after pretreatment with various fungi; where improved digestibility occurred, the fungi selectively used the hemicellulose rather than cellulose. Since the accessibility of cellulose by hydrolytic enzymes is inhibited by the presence of lignin, the digestibility of carbohydrates from the waste can be increased considerably by the decrease of its lignin content.

As the chemical delignification of wastes is too expensive for the fodder industry, the alternative way may be the conversion of lignocellulose by lignolytic microorganisms including several mushroom species. It has been reported by many research workers that mushroom cultivation which offers a practical option for producing food rich in protein and vitamins from wastes is the most economical means of utilizing lignocellulosic wastes (Bisaria *et al.*, 1987; Zadrazil and Reiniger, 1988).

Mushroom cultivation integrates the principles of microbiology, environmental technology, and solid - state fermentation in the transformation of waste materials into food for humans. Lignocellulosics can be recycled efficiently by mushroom cultivation (Fig.2.2).

Worldwide production of *Pleurotus spp.* (Oyster mushroom), *Auricularia spp.* (Wood ear), *Tramella fuciformis* (White jelly fungus or "silver ear") and *Pholiota nameko* ("Nameko" or Viscid mushroom) has increased at an accelerated rate of 112 -438% from 1986-1990 (Chang and Miles, 1991). The button mushroom (*Agaricus biosporus*), Shitake or oak mushroom (*Lentinus edodes*), straw mushroom (*Volvariella volvacea*) and winter mushroom (*Flammulina velutipes*) recorded a 16-43% increase over the same period.

The capacity of the lignin degradation by fungi is influenced by the growing tips of the filament that produce powerful extracellular lignin degrading enzymes, which act as "chemical drill" for penetrating the substrate and for converting lignin to metabolic products. Fungal mycelium attaches physically to lignin and agitation disrupts this contact. Therefore solid-state fermentation in thin layers is always preferred over agitated submerged fermentation as far as lignin degradation is concerned (Weiland 1988). Similarly, Ortega *et al.* (1993) showed that *Pleurotus ostreatus* and *Pleurotus spp.* grown in solid - state fermentation on sugarcane (baggase) degraded the dry matter by 50% after 60 days of fermentation. Both strains degraded the hemicellulose (about 80% of total degradation) and lignin (about 70%). The degradation of the lignocellulosic component of cotton stalks by *Pleurotus ostreatus* and subsequent increase in digestibility of the remaining organic matter has been described by Hadar *et al.*

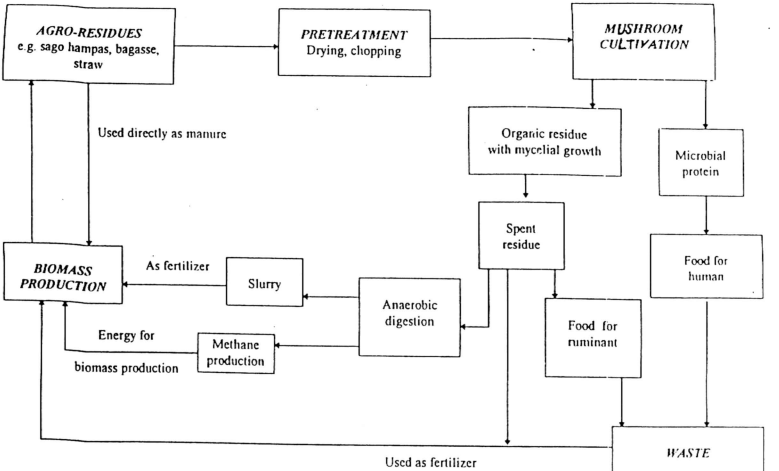

Fig 2.2: Recycling of agro-residues (adapted from Madan *et al.* 1987)

(1993). Other studies have reported the conversion of wood into feed by *Polyporus anceps*, *Ganoderma applantum*, *Phanerocheate chrysosporium*, *Polyporus versicolar* and *Fomitopsis ulmarius* (Reade and Mc Queen, 1983).

Pycnoporus sanguineus

Pycnoporus sanguineus (L, Murril 1904) a slow- growing saprophytic fungus belonging to the Basidiomycetes family Polyporaceae, is widely distributed on lignocellulosic substrate in tropical forests (Gazzanao, 1990; Almeida *et al.*, 1993) and is associated with aggressive white- rot type decay. This fungus has been long used in popular medicine by indigenous tribes of the Americas and Africa for treatment of number of illnesses and also by certain rural communities of the State of Santa Catarina, south of Brazil, for treatment of skin lesions (Smania *et al.*, 1995).

Cinnabarin is known to be its main orange colour antibiotic colouring component; but cinnabarinic acid and tramesanguin (Table 2.1) have also been shown to be among the minor constituents (Archenbach and Blumm 1991). The production of cinnabrin by three *P. sanguineus* strains was investigated and the antimicrobial activity of this substance against species from food sources was tested by (Smania *et al.*, 1998). The synthesis of antimicrobial compounds were evaluated in cultures of *P. sanguinues* MP 89007, occurring mainly between days 18 and 23 of incubation (Smania *et al.*, 1995).

Laccase appears to be produced as the sole ligninolytic enzyme by *P. sanguinues* in liquid culture and thus making it a good candidate as a fungus

Table 2.1: Constituents of *P.sanguineus* and their approximate concentrations.

[Content in % x 10^{-3}(dried *Pycnoporus* = 100%]

Compound class	Compound	Content
Sterols	Ergosterol	12.5
	5,6-Dihydroergosterol	11.2
	Ergosterolperoxide	6.2
Phenoxazine	Cinnabaribe	50.0
	O-Acetyl cinnabarine	1.2
	2-Amino-9-formylphenoxazone-1-carbonic acid	1.2
	9-Hydroxymethyl 1-2methylamino phenoxazone-1-carbonic acid methyl ester	0.9
	Phenoxazone	0.3
	Pycnosanguin	1.3

Source: Archenbach & Blumm (1991)

model. The tropical distribution of this fungus may also make it a particularly good candidate for use in the biotransformation of lignocellulosic wastes such as sago-hampas, cocopeat and baggase, which are produced in large quantities in tropical regions (Pointing *et al.*, 2000).

Azo and triphenylmethane dyes have widespread industrial use, yet they are not readily biodegradable. Partial decolorization of two azo dyes (orange G and amaranth) and complete decolorization of two triphenylmethane dyes (bromophenol blue and malachite green) was achieved by *P. sanguinues* cultures in submerged liquid culture producing laccase as the sole phenoloxidase (Pointing and Vrijmoed, 2000). According to (Schiephake *et al.*, 1993) *P. sanguineus* may also be suitable for such technology in decolorizing certain dye-containing wastewaters. *Pycnoporus sanguineus* was used in Kraft effluent decolorization with relative good results (Lambais, 1988; Esposoto *et al.*, 1991; Duran, 1992). Induction of phenoloxidase from *P. sanguineus* for use in effluent bioremediation as an alternative process to effluent detoxification has been repeated by Esposito *et al.* (1993).

Toxic pollutant like copper discharged from electroplating industry has a negative effect on the environment where it can accumulate through the food chain, thus posing a serious threat to human health. Various treatments have been introduced by Department of Environment Malaysia (DOEM), to reduce copper ions in wastewater. However, due to high cost of these methods, development of a more cost-effective system is necessary. Recently *P. sanguinues* has been studied to remove copper ions from an aqueous solution (Mashitah *et al.*, 1999).

Parkia oppositifolia, a grass from Amazon region of Brazil has been shown to be degraded by *P. sanguineus*. In the first stage non-selective degradation of wood components was observed. However, during the advanced decay, preferential cellulose degradation was observed (Castro *et al.*, 1993). These results were indicative that besides lignocellulotic enzymes, cellulolytic and xylanolytic enzymes were also produced. *Pycnoporus sanguineus* fungi were found growing on recycled plastic/wood composite lumber after only four years exposure in Florida (Morris and Cooper, 1998).

LIGNOCELLULOLYTIC ENZYMES

Microbial conversion of an abundant supply of lignocellulosic wastes (both from agriculture and forest) has become a subject of considerable interest as a renewable source of materials for fine chemical production, animal feedstuff, waste treatment and pharmaceutical manufacture (Destroy and Hasseltine, 1978; Buchholz *et al.*, 1980; Agosin and Odier, 1985 and Schiesser *et al.*, 1992). Lignin present in most lignocellulosics, acts as an obstacle to microbial utilization of cellulose and hemicellulose in such materials. Exploitation of the potential of the lignocellulosic biomass requires that each of the major polymers namely lignin, cellulose and hemicellulose be utilized, perhaps by conversion to their simpler constituents and then to more valuable end- products.

Biological processing offers many potential advantages over conventional chemical and/or physical processing methods. Bioconversion has received many attention as it emulates nature where lignocellulosics are recycled on a vast scale by various microorganisms, notably by the lignin degrading basidiomycete fungi,

thus increasing the dry matter digestibility (Zadrazil, 1977,1985; Reade and McQueen, 1983; Moyson and Verachtert, 1991). However, such processes must be efficient to be cost effective. Thus a thorough understanding of the enzyme systems required for the hydrolysis of each of the major components in question and of the microorganisms that produce these enzyme systems is prerequisite.

The knowledge of the types of enzymes produced by basidiomycete fungi when growing on lignocellulosic substrate is of considerable value in designing procedures to modify or improve existing processes using these fungi (Wood *et al.*, 1988). Such processes include the production of edible fungi (mushroom), animal feed, enzymes, paper- industry-waste processing and other possible by-products. Table 2.2 summarises some extracellular enzymes from basidomycetes and their mode of action on lignocellulose.

The major function of such extracellular enzymes is to break down the complex carbohydrates which not only provide nutrition for the fungus but they may also have additional functions such as regulating morphogenesis and in the production of antimicrobial systems. Recognition of the types of enzymes produced for lignocellulose degradation will allow the development of several strategies for strain improvement including mutation and selection, gene cloning and transformation (Wood *et al.*, 1988).

Recovering enzymes from white-rot fungi grown on lignocellulose could offer the opportunity to produce a powerful set of enzymes for research and development, which are uniquely capable of carrying out a range of extensive or highly- specific modifications to the lignocellulose.

Table 2.2 : **Extracellular enzymes from basidomycetes and mode of action in lignocellulose biodegradation**

Name	Reaction
Lignin peroxidase	Cα-Cß cleavage of propyl side chains of lignin, lignin model compounds, partially depolymerises lignin.
Laccase	Oxidizes σ- and *p*-phenols and aromatic amines to quinines.
Endocellulase	Cleavage of internal 1,4 ß-Dglycosidicbonds in cellulose chains.
Exocellulase	Removal of cellobiose units from non-reducing ends of cellulose chains.
ß-glycosidase	Cellobiose hydrolysis to glucose units.
Xylanase	Cleavage of internal 1,4 ß-Dglycosidicbonds in xylans.

Summarised from Wood *et al.* (1988)

The ligninolytic enzymes, lignin peroxidase (LiP; EC 1.11.1.14) and manganese dependent peroxidase (MnP; EC 1.11.1.13) are produced by *Phanerochaete chrysosporium* (Kirk and Farrell, 1987). Recently Srinivasan *et al.* (1995) demonstrated that *Phanerochaete chrysosporium* BKM-F1767 did produce extracellular laccase (EC 1.10.3.2) in a defined medium, contrary to the widely held belief that this fungus does not produce laccase (Kirk and Farrell, 1987; Thurston, 1994). *Lentinula edodes* secrete a range of important degradation enzymes (Leatham *et al.*, 1991). Lignin peroxidase has not been reported in *Pleurotus spp.* (Fu *et al.*, 1997). These fungi produces laccase and Mn-dependent peroxidase (Gutierrez *et al.*, 1994). Laccase is reported to oxidize a wide range of substituted phenols (Shuttleworth and Bollag, 1986).

The natural function of laccase from white rot fungi is not known although it seems to have a role in fruiting (Wood, 1980). It is possible that laccase renders phenolic compounds less toxic though the polymerization reaction of wastes would be to decrease chemical consumption/dependence and reduce environmental loading. The recent discovery of these ligninolytic enzymes, that are thought to have significant role in degradation of lignin, has led lignin biodegradation research onto practical industrial applications such as biopulping and biobleaching (Kumaran 1996). Thus producing and characterising microbial degradative enzymes has important commercial applications or supply information that permits the most effective use or improvement of microbial cultures.

Lignin degrading enzymes

The processes involved in lignin degradation revolves around three main classes of ligninolytic enzymes i.e. lignin peroxidase, manganese-dependent peroxidase and laccase (Kirk and Farell, 1987; Higuchi, 1993).

Lignin peroxidase was reported for the first time in 1983 as an extracellular oxygenase (lignonase) produced by the white-rot fungus *Phanerochaete chrysosporium* (Glenn *et al.*, 1983; Tien and Kirk 1983). The enzyme was later identified as a peroxidase. The discovery was preceded by a study of a Cα-Cβ cleavage reaction in a dimeric lignin model by lignolytic cultures. The activity responsible for this reaction was identified as a single protein having a molecular weight of 42 Kda and a PI of 3.5. Lignin peroxidase is the most potent enzyme having the capability to oxidize substrates with high potential ionization (Kersten *et al.*, 1990). This enzyme uses small aromatic fungal metabolites such a veratryl alcohol and 2 chloro-1, 4-dimethoxybenzene as a cofactor. LiP oxidizes nonphenolic lignin structures by removing the electron and generating cation radicals, which are then decomposed chemically.

Glen and Gold (1985) found another type of peroxide in *Phanerochaete chrysosporium* which is Mn^{2+} dependent. This enzyme oxidizes Mn (II) to Mn (III), which then oxidizes phenolic compounds to phenoxy radicals. This leads to decomposition of the lignin substrates. MnP appears to function as phenol-oxidizing enzymes and perhaps participates in H_2O_2 production.

Both are heme-containing glycoproteins containing 20-30% sugar requiring hydrogen peroxide as an oxidant. Their molecular weight is around 40,000. They have rather acidic PI's ranging from 3.2 to 4.9. These peroxidases have catalyzed the initial oxidation of wide variety of chemicals including structurally diverse (Banat *et al.*, 1996; Buckley and Dobson, 1998).

Laccase is one of the few enzymes that has been the subject of study since the end of the last century. The enzyme is a copper - containing polyphenol oxidase that was discovered in the exudates of the Japanese lacquer tree *Rhus vernicefera* (Yoshida, 1883) and subsequently was demonstrated as a fungal enzyme as well. At present, there is only one bacterium, *Azospirillum lipoferum*, in which laccase type polyphenol oxidase has been demonstrated (Diamantidis *et al.*, 2000). Laccase (benzenediol: oxygen oxireductases [EC 1.10.3.2]) is a family of multi copper oxidases containing enzymes catalyzing the oxidation of a broad number of phenolic compounds and aromatic amines by using molecular oxygen as the electron acceptor, which is reduced to water (Reinhammar and Malastrom, 1981). Lacccase can oxidize non-phenolic compounds with a relatively low ionization potential.

Fungal laccases, in addition to being related to different physiological processes (Thurston, 1994) are involved in lignin degradation together with lignin peroxidase (LiP) and manganese peroxidase (MnP) (Higuchi, 1990). They are secreted by most of the basidiomycetes that cause white-rot of wood (Hatakka, 1994) and may play a role in ligninolysis by these fungi (Kawai *et al.*, 1988). Laccase in woody tissues is a component of the lignin synthesizing system (Bao

et al., 1993). The typical laccase is 60-80 Kda molecules of which 15-20% is carbohydrate and the measured copper content of the purified laccase varies between four and two atoms per enzyme molecule (subunit) (Thurston, 1994).

Recently, laccase and other lignocellulose modifying enzymes have been reported in marine fungi (Ascomycetes) which are often found on decaying branches, leaves and driftwood (Raghukumar *et al.*, 1994). The presence of laccases in a number of marine fungi tested suggested that these organisms play a lignin modification in the marine environment. Although the role of laccase in lignin degradation is still unclear, it was recently suggested that the enzyme plays a more important role than previously thought. Using such lignin model compounds, it was found that the enzyme from *C. versicolor* catalyzed cleavage of side chains and non-phenolic bonds, and opening of aromatic rings these are important reactions in depolymerisation of the lignin (Kawai *et al.*, 1988; Bourbonnais and Paice, 1990 and Higuchi 1993).

There is little evidence that laccases by themselves can catalyze ligninolysis, but they are able to depolymerize synthetic lignin (Kawai *et al.*, 1999) and delignify wood pulps (Bourbonnais *et al.*, 1997; Call and Muckle, 1997) when they are combined with various low molecular weight electron transfer agents. One of the most intensively researched mediators is 1-hydroxybenzotriazole (HOBT), which is oxidized to its nitroxide radical by laccase (Bourbonnais *et al.*, 1998; Potthast *et al.*, 1999). It also degrades phenolic model compounds that represent condensed kraft lignin structures such

as biphenyls, stilbenes, and diphenylmethanes (Xu *et al.*, 1997; Crestini and Argyropoulos, 1998). These reactions are undoubtly important, because the phenolic substructure content of residual softwood Kraft lignin is generally 25-50% (Francis *et al.*, 1991; Lachenal *et al.*, 1999).

It has been postulated that laccase is involved in various cellular and microbial activities. Recent studies on the physiological function of laccase include those on plant cell wall biosynthesis (O'Malley *et al.*, 1993), phytopathogenesis (Sbaghi *et al.*, 1996), wood material degradation and humification (Machuca and Duran, 1993;) insect sclerotization (Anderson *et al.*, 1985; Barett 1987; Thomas *et al.*, 1989 and Miessner *et al.*, 1991), bacterial melanization (Faure *et al.*, 1994), and melanin-related virulene for human (Williamson, 1994). As an oxidase, laccase may be used in many industrial and medicinal applications where oxidation or oxidative derivatization is involved. Currently, investigations are focused on laccase-based biooxidation, biotransformation, biosensor and enzymatic synthesis (Michael and Stephen, 1999).

Xylan degrading enzymes

Xylan is the most abundant noncellulosic polysaccharide present in both hardwoods and annual plants, and accounts for 25 -35% of the total dry weight in tropical plant biomass. In temperate softwoods, xylans are less abundant and may compromise about 8% of the total dry weight. It is a heteropolymer with a backbone of - β1, 4-D-xylopyranosyl residues and branches of neutral and uronic

monosacharides and oligosachrides. In simplest forms xylans are linear homopolymers that contain D-xylose monomers linked through β1, 4-glycosly bonds. In nature, they are partially substituted by acetyl 4-o-methyl-D-glucuronosyl and L -arabinofuranosyl residues, forming complex heterogeneous and polydispersed polymers. Many structural aspects of xylans are unclear because of the difficulties associated with the isolation of xylans from natural raw materials without significant alteration or loss of the original structure and association with other components.

Xylanases from various fungi and bacteria have been isolated and characterized. Although xylanases are produced by fungi, yeast and bacteria, filamentous fungi are preferred for commercial production as the levels of the enzyme produced by fungal cultures are higher then those obtained from yeast or bacteria. Fungal xylanases are often acidic to neutral in nature, and xylanases from thermophilic fungi are relatively more thermostable (Nissen *et al.*, 1992). On the other hand, bacterial xylanases range from acidic to alkaline and from moderate to extremely thermostable depending on the type of organism. Most organisms produce multiple forms of xylanases, the multiplicity of the xylanases is suggested to be required for all effective action on xylans in which all xylosidic linkages are not equivalent and equally accessible (Wong *et al.*, 1988). Recombinant DNA technology is being utilized as a tool to produce better understanding of xylanases (Coughlan and Hazlewood 1993).

In many microorganisms, xylanase activity has generally been found in association with cellulases, β-glucosidase or other enzymes, although there are

many reports that have described in SSF systems, production of cellulase-free and other enzyme- free xylanase.

Most of the xylanases characterized act randomly and are able to hydrolyze different types of xylans, showing only differences in the spectrum of end products. The main products formed from the hydrolyses of xylans are xylobiose, xylotriose and substituted oligomers of two or four xylosyl residues.

Application of xylanases together with other bleach agents, such as oxygen and hydrogen peroxide in pulp industry has been extensively investigated and projections of a totally chlorine-free technology has been put forward (Dutton, 1992). Pulpzyme introduced by Novo Nordisk A/S, was the first commercially available xylanase for use in biobleaching of pulps. It was extracted from a strain of *Trichoderma reesei* and was used in the first bleaching stage to remove the dosage of active chlorine.

Among the xylanolytic enzymes, xylanases (endo-1, 4-β-D-xylan xylanohydrolase; EC 3.2.1.8), which degrade the xylan backbone into small oligomers, are the most abundant components and the main enzymes required in most application such as bleaching of kraft pulp, increasing the brightness of pulp, improving the digestibility of animal feed, clarification of juices, etc (Javier *et al.*, 1998).

Cellulose degrading enzymes

Cellulose is a linear polymer of glucose in plant and woody materials. It is associated with hemicellulose and other structural polysaccharides, and surrounded by a lignin seal. The main characteristics of cellulose are its great

strength, fibrous nature, insolubility, and inertness. Cellulose is basically composed of long, linear chains of β-1, 4 linked glucose units (Wood, 1989).

Cellulases are enzyme complexes, which in stepwise, breakdown cellulose or derivatives of cellulose to glucose. According to Wood (1989) and Gerhartz (1990), cellulose depolymerization needs a synergistic reaction of three hydrolytic enzymes and the first attack is simultaneously affected by at least two of them. The three enzymes involved in cellulose breakdown are (a) exocellulase or exobiohydrolase (EC 3.2.1.91), (b) endocellulase or endoglucanase (EC 3.2.1.4) and (c) β- glucosidase or cellobiase (EC 3.2.1.21).

The primary step in cellulose hydrolysis is the degradation of some amorphous regions in the cellulose fibers by endoglucanase. New free end-chains of cellulose are produced which then becomes the substrate for exocellulose (also called C_1 activity). This enzyme removes cellobiose from the non-reducing end of the polyglucan chain. By this way, the substrate for the action of endoglucanase (often called C_X activity) is formed. The third enzyme, β-glucosidase hydrolyses cellobiose released during this sequence of reactions. This step is crucial because the accumulation of cellobiose inhibits cellulose degradation by the other enzymes. C_X cellulose degrades soluble fragments or derivatives of cellulose (carboxymethyl cellulose or hydroxyethyl cellulose), forming cellobiose. It is reported that the effect of enzymatic hydrolysis of cellulose depends very much on the pretreatment (temperature, alkaline oxidation and mechanical degradation) of the substrate (Olsen, 1995).

SOME APPLICATIONS OF LIGNOCELLULOLYTIC SYSTEMS

Potential applications in lignocellulose conversion are especially likely to require multiple enzymes with specificities reflecting the complexity of lignocellulose and the conversion desired. Such applications could be more easily developed if researchers had better access to a large range of useful enzymes (Leatham *et al.*, 1991).

The characterization and use of fungal degradative enzymes produced on solid substrates is a promising area that merits more research. Besides helping to understand lignocellulose degradation the information on fungal degradative enzymes could be used to develop a range of new commercial applications.

The most important application for a multi- enzyme set produced by the SSF method would perhaps be in efficient fuel and chemical feedstock production, improving (paper fibrillation, water retention, increased freeness in recycled fibres, and selective removal of xylan from dissolved pulps) and increasing animal feed digestibilities (Zadrazil *et al.*, 1996). Table 2.3 lists some enzyme involved in biomass conversion. In addition, the potential application based on ligocellulose conversion include the bio pulping of wood, bioremediation and biodegradation of hazardous compounds, biological detoxification of agro-industrial residues, biotransformation of crops and crop residues (Pandey *et al.*, 2000), aerobic reduction of waste cellulose and other wood-derived materials prior to land-filling (Leatham *et al.*, 1991). Similarly, in

Table 2.3: Enzymes involved in the conversion of biomass

Cellulose - degrading enzymes	Cellobiohydrolase Endo-1, 4 – β - glucanase β – Glucosidase
Hemicellulose- degrading enzymes	endo-1, 4 – β – xylanase endo-1, 4 – β – mannanase β – xylosidase β – mannosidase α – arabinosidase α – Galactosidase α – Glucoronidase Acetyl esterase
Pectin- degrading enzymes	Endopolygalacturonase Exoplygalacturonase Pectinesterase Pectinlyase Pectatelyase
Lignin- degrading enzymes	Oxidases Dehydrogenases Peroxidases

Source: Linko *et al.*, (1989)

the feed industry, treatment of silage with enzymes aims at improved digestibility and the feed conversion ratio (Linko *et al.*, 1989). The sugar produced as the result of enzyme hydrolysis may be partly converted to lactic acid which lowers the pH sufficiently to ensure stability of the silage without addition of acids (Linko *et al.*, 1989).

SOLID STATE FERMENTATION

Solid substrate fermentation should be used to define only those processes in which the substrate itself acts as carbon / energy source, occurring in the absence or near- absence of free water: Solid state fermentation should define any fermentation process occurring in the absence or near- absence of free water, employing a natural substrate as above, or an inert substrate used as solid support (Pandey *et al.*, 1999). In literature, the two terms, solid-state and solid-substrate fermentation have been loosely used to refer to the same process, the distinction between a solid-state fermentation and solid-substrate fermentation is difficult to define precisely as free water contents of the water varies widely (Doelle, 1994), e.g. free water in maple bark is 40% as moisture, and in sago 'hampas' is 90% as moisture.

Such processes are used on a commercial scale for the production of different types of fermented foods, particularly in the Orient and in some developing countries. In addition to their use in food production, SSF's have been used successfully in recent years for large scale productions of fungal metabolites and for the biomass and enzymes (Aidoo *et al.*, 1982; Moo-Young *et al.*, 1983) Fig. 2.3 outlines the principal process steps in SSF. There are several important

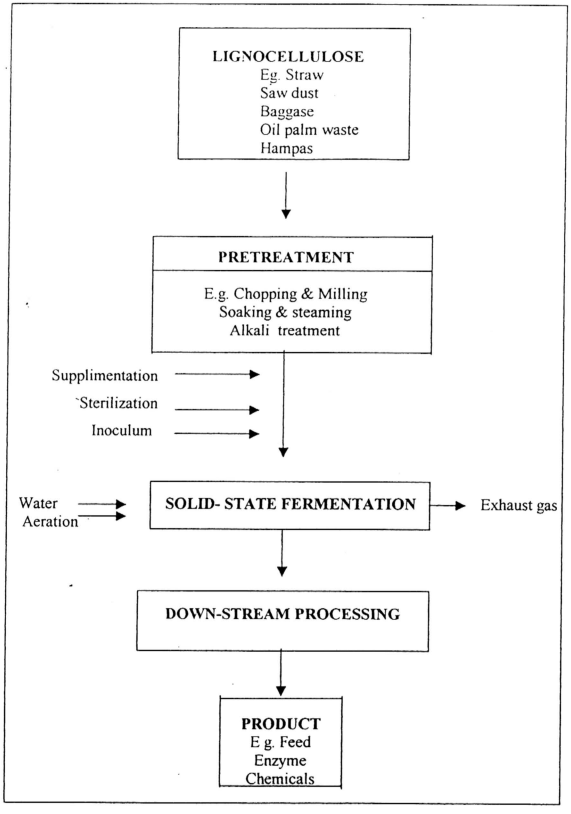

Fig: 2.3 Principal steps in solid-state fermentation

factors, which affect SSF processes. Among these, selection of a suitable strain, substrate and selection of process parameters (physical, chemical and biochemical) are crucial. While efforts largely continued to exploit filamentous fungi and yeast for the production of various products, attempts also have been made to explore the possibilities of using bacterial strains in SSF systems. Enzyme production has been an area in which several bacterial strains have been used and success have been achieved (Pandey and Soccol 1998; Soccol and Krieger 1998; Pandey *et al.*, 1999 and Pandey *et al.*, 2000).

Among the filamentous fungi Phycomycetes (*Mucor and Rhizopus*), Ascomycetes (*Aspergillus and Pencillium*) and Basidiomycetes (white-rot fungi) continue to be the most preferred choice as they are ' ideal microorganisms' capable of utilizing most of the polymers in lignocellulosic residues (Kirk, 1983). They are known to excrete a range of hydrolytic and oxidizing enzymes into the ligocellulosic substrate to depolymerize the lignocellulose polymers which can be assimilated by the fungi (Wood *et al.*, 1988). Solid surfaces is typical property of filamentous fungi that decay organic matter therefore SSF processes are closeness to the natural way of life of filamentous fungi.

The selection of a substrate for SSF process depends upon several factors mainly related with cost and availability and thus may involve screening of several agro-industrial residues, the solid substrate not only supplies nutrients to the microbial culture growing in it, but also serves as an anchorage for the cells.

The substrate that provides all the needed nutrients to the microorganisms growing in it should be considered as the ideal substrate. However some of the nutrients may be available in sub-optimal concentrations, or not present in the substrates. In such cases, it would be necessary to supplement them externally.

It also has been a practice to pre-treat (chemically or mechanically) some substrates (e.g. ligno-cellulosics) which makes them more easily accessible for microbial growth. Generally, smaller substrate particles would provide larger surface area for microbial attack and thus should be considered as a desirable factor. However, too small substrate particles may result in substrate aggomulation in most of the cases which may interfere with microbial respiration / aeration, and thus may result in poor growth. At the same time, larger particles provide better respiration / aeration efficiency (due to increased inter -particle space) but provide limited surface for microbial attack. Thus, it would be necessary to arrive at a compromised particle size for a particular process (Pandey *et al.*, 2000).

Moisture content is the key parameter for regulating and optimizing SSF processes, because the moisture content of the substrate influences the fungal growth, the enzyme activity and the accessibility of the substrate and regulates product formation too. Furthermore moisture content in the pores influences the mass transfer rate of oxygen and carbon dioxide and the rate of heat dissipation (Weiland, 1988). Microbial growth and product formation occurs at or near the surface of the solid substrate particles having low moisture contents. Thus, it

is crucial to provide an optimized water content, and control the water activity (a_w) of the fermenting substrate; the availability of the water in higher or lower concentrations affects microbial activity adversely. Moreover, water has profound impact on the physico- chemical properties of the solids and this, in turn, affects the overall process productivity.

Since no free water is present, the volume of the media per gram of the substrate is drastically reduced (Cannel and Moo Young, 1980). This leads to many obvious benefits:

i. The space taken up by the fermentation vessel is small relative to yield of product because less water is used and the substrate is concentrated.

ii. Significantly, the low moisture required to get maximum yields of the product with fungi excludes any problem of contamination by fast growing bacteria which grow on solid substrates only at moisture levels above 50% (Weiland, 1988). Eliminating this possibility is certainly one major advantage of the technique.

iii. The conditions under which the fungus grows are more like those found in its natural habitat.

iv. The desired product may be readily extracted from the vessel by adding the solvent directly. In doing so less solvent is required.

v. If one does not wish to extract the product immediately, the substrate may be placed in plastic or in paper bags and frozen

without having to store large volumes of liquid in breakable bottles or tie up large metal tanks.

vi. Since the product is concentrated, it may be dried and incorporated directly into animal feeds

Pandey *et al.* (2000) stated that selection of a suitable substrate has mainly centered around tropical agro-industrial crops and residues. Due to their rich organic nature, these can serve as ideal substrates for microbial processes for the production of value-added products. Solid-state fermentation has been mostly employed for the bioconversion processes. Application of agro-industrial residues in bio processes, on one hand, provides alternative substrates, and on other, helps in solving pollution problems, which otherwise may cause their disposal. Many processes have been developed that utilize these agro-industrial residual raw material for the production of bulk chemicals and value- added fine products such as ethanol, single-cell protein (SCP), mushrooms, enzymes, organic acids, biologically active secondary metabolites, etc.

Sterility is very often required in any submerged fermentation (SmF) process because many contaminants could complete the process organism under the conditions of high water availability provided. Often SSF processes involve an organism which grows quite rapidly under the low water conditions. If an active inoculum is added to a (cooked) substrate, the process organism is able to out compete contaminating organisms, meaning that strict aseptic operation of the bioreactor may not be essential in SSF, although, of course,

operation should be carried out in as clean a manner as possible. The less stringent design requirements for such bioreactors, and correspondingly lower costs, could be considered as a favorable point for the SSF process.

Moo Young *et al.* (1983) stated that the solid - state fermentation techniques have the advantages of low capital cost and low energy expenditure. The relatively dry substrate produce low wastewater and an absence of foaming during the process.

As solid-state fermentation process is essentially a compost, self- heating occurs with the result that heat removal may pose a problem in large scale operation. However, overheating can usually be prevented by forced aeration (Cannel and Moo Young, 1980; Moo Young *et al.*, 1983).

Cannel and Moo Young (1980) stated that the types of microorganisms that can be used in solid-state fermentation are limited to those that can grow at reduced moisture levels (low water activity), namely fungi, some yeasts and streptomycetes. The intra-particle mass transfer is usually the rate-limiting step in solid-state fermentation. Process control such as measurement of moisture levels, pH, oxygen levels, carbon dioxide levels and product yield is more difficult in solid-state fermentations.

According to Aidoo *et al.* (1982), substrate used in solid-state fermentation may have to be pretreated by pearling, cracking or dehulling before use. During the process, there is difficulty in estimation of mycelial biomass. In case where spore inoculum is used, the size of inoculum needed is presumably quite large.

It is important to recognize that each microbe - substrate system is unique and must be considered in terms of chemical composition and physical properties of that of the substrates, the growth characteristics and physiology of the organism cultured and the nature of the product. Mudgett (1986) suggested a number of key variables including pretreatment, supplementation, particle size, moisture content, sterilization, inoculum density, temperature, pH, agitation and aeration which should be considered in process development of solid substrate fermentations.

Supplements like mineral media and nitrogen salts are incorporated to initiate biomass production, induce enzyme syntheses, provide balanced growth conditions, or prolong secondary metabolite production. For instance, for several brown-rot fungi, lignin degradation does not provide enough energy for enzyme induction and utilization of cellulosic constituents. A supplemental carbon source may be required for growth and further lignin degradation, i.e., co-metabolism. Similarly, the white- rot fungi *P.chrysosporium* and *T. versicolor* are enabled to degrade spruce lignin to a significant extent without a cometabolite such as cellulose or glucose for growth. Supplementary nutritional requirements for a developmental microbe substrate system of interest may be determined by preliminary experiments in either submerged or solid-state cultures at the laboratory scale.

Water is a biological material which generally exists in three states, as determined by moisture isotherm measurements in which the solids sorb and desorb water vapour in equilibrium with relative humidities (water activities) in

a gas phase which can be maintained by saturated salt solutions at a constant temperature. Hesseltine (1965) commented that one of the secrets to successful solid-state fermentation is having the substrate moist enough for mold growth, but not so wet that bacterial growth is promoted. In view of such factors, the optimum moisture content for each microbe - substrate system should be determined based on the desired product and the conditions for cultivation.

It is generally necessary to optimize inoculum density in solid-state fermentations. If the density is too low it may give insufficient biomass and permit the growth of undesirable organisms. On the other hand, If the density is too high it may produce too much of biomass and deplete the nutrients of the substrate that is necessary for product formation.

The temperature and pH of the fermenting solids are significant variables in solid-state processes and are generally specific to the organisms and the product as in SmFs. Both are difficult to control in the solid state, since direct measurements in the liquid phase are impractical because of their association with solids at low moistures. Optimal temperatures for growth may not be the same as for product formations, suggesting a possible need for temperature shifts (profiling) in later stages of fermentation. The rate of heat generation in the bed at high levels of biological activity may induce thermal gradients within the bed which lead to the transfer limitations and sub optimal conditions for biomass or product formation.

At solid surfaces near which measure biological activity occurs, the bulk pH of the liquid phase may be considerably different from local pH levels.

This is due to surface charge effects and ionic equilibria modified by solute transport effects.

Mixing of the solid substrate promotes the interparticle mass and heat transfer which results in more uniform conditions. It improves oxygen supply to the microorganisms and the carbon dioxide removal from the void spaces of the solid substrate. Mixing may be beneficial for continuous operation, automatization and scale-up of SSF processes. On the other hand, mixing influences the morphology of the microorganisms, which may result in a change of metabolism, lower product yields or a total failure of SSF process, if the shear forces breakup the growing tips of the mycelial hyphae. The stress sensitivity of mycelia is strongly dependent on the type of microorganisms used and the morphology of the filaments. Since stress sensitivity of filamentous fungi, especially of fungi growing on solid substrates, is rather unknown, the applicability of agitated or turned SSF fermenters for delignification must be tested in each individual case (Weiland, 1988).

SOLID- STATE FERMENTATION FOR ENZYME PRODUCTION

Enzymes are among the most important products obtained for human needs through microbial sources. A large number of industrial processes in the areas of industrial, environmental and food biotechnology utilize enzymes at some stage or the other. Current developments in biotechnology are yielding new applications for enzymes. SSF holds tremendous potential for the production of enzymes (Pandey *et al.*, 1999).

Ideally, almost all the known microbial enzymes can be produced under SSF systems. Literature survey reveals that much work has been carried out on the production of enzymes of industrial importance like proteases, cellulases, ligninases, xylanases, pectinases, amylases, glucoamylases etc. Attempts are also being made to study SSF processes for the production of inulinases, phytases, tannases, phenolic acid esterases, microbial rennets, aryl - alcohol oxidases, oligosaccharide oxidases, tannin acyl hydrolase, α- L - arabinofuranosidase, etc. using SSF systems (Pandey et *al.*, 1999).

It can be of special interest in those processes where the crude fermented product may be used directly as enzymes source. In addition to well-established applications in the food and fermentation industries, microbial enzymes have attained a significant role in biotransformations involving organic acid media, mainly for bioactive compounds. Table 2.4 lists some of possible application of enzymes produced in SSF.

Currently, industrial demands of most of the enzyme is met by production using submerged fermentation (SmF), generally employing genetically modified strains. The cost of production in SmF is high and it is uneconomical to use many enzymes in several processes. This necessitates reduction in production cost by alternative methods. SSF should be considered an attractive alternative. Tengerdy (1998) advocated that SSF was particularly suitable for ligno-cellulosic enzyme production for various agro- biotechnological applications. To illustrate this, a comparison was made for cellulase production in SSF and SmF. In SmF, cellulase yields are generally about 10 g / l, and the average fermentation cost in a stirred tank bioreactor is about \$200 / m^3. Thus, the production cost in the crude

Table: 2.4: Industrial applications of enzymes produced by SSF.

Process	Enzyme
Enzyme-assisted ensiling	Fugal cellulases and hemicellulases
Bioprocessing of crops and crop residues	Fugal cellulases and hemicellulases
Fibre processing (retting)	Fungal pectinases, cellulases, hemicellulases
Feed supplement	Amylases, proteases, lipases, cellulases, hemicellulases
Biopulping	Xylanases
Direct compositing	Hydrolytic enzymes
Soil bioremediation	Laccases, ligninases
Post-harvest residue decomposition	*Trichoderma harzianum* cellulases
Biopesticide	*T.harzianum* cellulase for helper function

Source: Pandey *et al.* (1999)

fermentation by SmF is about $20/kg. In SSF, the average production level is about 10 mg /g substrate and the average fermentation cost is only about $25/mt. Thus, the unit cost of SSF cellulase is just about $0.2/kg (Tengerdy, 1996; Tengerdy, 1998).

A thermostable β- galactosidase was reported from a thermophilic *Rhizomucor sp.* Enzyme activities by SSF were 9-fold more than by SmF processes (Shaikh *et al.*, 1997). Laptane and Chahal (1993) compared ligninase production in SSF systems and SmF system using a culture of *P. chrysosporium* ATCC 24725. Higher yields of ligninases, especially laccase and Mn-peroxidase, were obtained in SSF system. Hata *et al.*, (1997) compared the two glucoamylases produced in SSF and SmF systems using *A. oryzae*. Enzyme produced by SSF could digest raw starch but that by SmF could not. Archana and Satyanarayana, (1997) described a SSF process for the production of thermostable xylanase by thermophilic *Bacillus licheniformis*. Enzyme production was 2-fold higher in SSF system than in SmF system.

It is interesting to note down that although a number of substrates have been employed for cultivating different microorganisms, wheat bran has been the preferred choice in most of the studies. Malathi and Chakraborthy (1991) evaluated a number of carbon sources (brans) for the alkaline protease production and reported wheat bran to be the best for cultivation of *A. flavus* IMI 327634.