Introduction

The Indian pulp and paper industry is over a hundred years old. First mill in the country was commissioned in 1812 in Serampur (West Bengal). However, the growth of the industry has been uneven and as a result, the Indian paper industry is a mix of large integrated plants based on wood based raw material and medium and small size paper plants based on waste paper. The capacities of the mills range from 500 tonnes/annum to 2.0 lakh tonnes/ annum. There are about 700 units, which manufacture pulp, paper, paperboard and newsprint paper, out of which nearly 570 are in operation. Based on the raw material utilized, the paper units can be classified into three broad categories as Wood based (Bamboo, hardwood), Agro-based (Bagasse, jute, rice & wheat straw) Waste paper based.

Pulp and paper industry is considered to be one of the most water and energy consuming industry in the world as it uses approximately 4% of total energy used worldwide and also considered as one of the most polluter industry according to Thompson *et al* 2001 and Sumathi and Hung 2006. Raw material preparation, Pulp manufacturing, Pulp Washing and Screening, Chemical recovery, Bleaching, and Papermaking are the main steps in pulp and paper manufacturing process (Bajpai 2012).

The process of papermaking result in large volumes of wastewaters, which contain dissolved wood-derived substances and residual process chemicals. Owing to the increased environmental awareness, these potentially highly polluting wastewaters generated from the mills cannot be directly released in nature. Instead, recycling of the wastewaters is becoming an attractive alternative for many pulp and paper mills as it also offers potential savings in the cost of fresh water. Recycling of the wastewaters

can be done either by closing up the systems in the mill or by treating the wastewaters such that they can be reused.

Wastewater purification is carried out by sequential methods. The first step involves a primary clarification by sedimentation or flotation in order to remove solid materials. The secondary treatment involves either an aerobic microbial process called the activated sludge operation, or an anaerobic digestion. The activated sludge process is the most commonly used approach for treating the waste water in pulp and paper mills, operates through successive action of many different microbes active in the sludge. The process is often carried on site in large aerated tanks. The success of the process is dependent on successful maintenance of the dissolved oxygen as well as good settling of the sludge. The settlement also depends on the type of microbial flora involved. However, as the growth requirements are different for different microbial species, these problems can often be controlled by varying the rate of aeration, temperature or the nutrient composition of the process (Thompson *et al* 2001). In biological waste treatment a very large number of different microbes can be used. Sometimes additional nutrition, as nitrogen in the form of urea, is added to enhance the biological waste treatment.

Discussed below is a brief description on the traditional method of making paper practiced by most of the paper industries.

PROCESS OF PAPER MAKING

Process of paper making includes the following different stages.

Raw materials and its processing

Pulp and paper are manufactured from raw materials containing cellulose fibers, generally wood, recycled paper, and agricultural residues. About 60% of cellulose fibers originate from nonwood raw materials in developing countries,

nonwoody materials mostly used are bagasse, cereal straw, bamboo, esparto grass, jute, flax, and sisal (Gullichsen 2000).Conifers are also sometimes preferred as their fibers are longer than, fibers of deciduous trees. Conifers used are mainly spruce, fir and pine, whereas beech, birch, poplar and eucalyptus are the most important deciduous varieties used for paper (htpp: //www.sappi.com). Raw material preparation is the initial process of manufacturing pulp which starts with debarking, when wood is used as raw material (Smook 1992 a, Bierman 1996a).

PULPING

Pulping process can be differentiated in to two major types: mechanical pulping and chemical pulping. Figure 1 represents an over view of pulping process. From the raw material wood, cellulosic pulp is prepared which has been pretreated by chemical or mechanical means (or a hybrid of these two), and then processed into a range of products, including different grades of paper and packaging materials, depending on pulp quality (Grönqvist *et al* 2003).

Mechanical Pulping:

In mechanical pulping process separation of fibers is done by using mechanical energy applied to the wood matrix causing the gradual break of the bonds between the fibers and release of fiber from the wood matrix (Smook 1992b, Biermann1996b). The main processes of mechanical pulping are Stone Ground wood Pulping (SGW), Pressure Ground wood Pulping (PGW), Refiner mechanical pulping (RMP), Thermo-Mechanical Pulping (TMP), or Chemi-Thermo-Mechanical Pulping (CTMP).

The first process used for making paper from wood is Ground wood pulping. In this pulping process grinding of wood into pulp by pressing wood against a quickly rotating stone to pull out the fibers under addition of water so continuous washing of

fibers is carried out. Due to the friction high temperature is generated in the refining zone which helps in softening of wood. The yield is high (about 95%) the fibers are stiff and bulky because most of the lignin remains. Paper produced from ground wood pulp has low strength and high color reversion, but the opacity is excellent. Though Ground wood pulping is an energy-intensive process it has been used to make magazine papers (Arppe 2001).

In the RMP process wood chips are processed through a rotating disk refiner. The refiner plate is made up of three different zones first to break the chips, then to produce intermediate size fragments, and finally to produce single fibers. This produces fibers with better bonding properties, and thus better paper strength than SGW pulp. However, opacity is reduced, color reversion is similar, and the energy expenditure is increased compared to SGW.

The TMP process is a modification of the RMP process in which involved steam pretreatment at 110–150°C to soften the wood and followed by refining. In the refining process initially the refiners are kept at high temperature and pressure to promote liberation of fibers and later on the refiners are kept at ambient temperature to treat the fibers for papermaking. The higher temperature during refining helps in softening of the fibers and maintaining the length of fibers in the recovery process.

Chemimechanical processes involve mild chemical treatment before applying mechanical pressure. In this process wood chips undergo treatment of buffered sodium sulfite solution, and thentreated in disk refiners to complete the fiber separation. The sulfonation of middle lamella lignin causes a partial dissolution so that the fibers are weakened for the subsequent mechanical defibration.

In Thermomechanical process, heat is used in addition to mechanical operations that involve high-temperature steaming before refining which softens the

interfiber lignin and exposes cellulosic surfaces. Chemimechanical pulping and chemithermomechanical pulping (CTMP) are similar but use less mechanical energy and soften the pulp by use of chemicals like sodium sulfite, carbonate, or hydroxide (Bajpai 2012). Mechanical pulps are particularly used for printing-grade papers because it retains an excellent optical properties (reflectivity, specific light scattering coefficients and specific light absorption coefficients) (Pere, Matti and Viikari 2000) and mechanical pulp is used for newsprint and coated printing grades where it provides a well-filled and formed sheet. In fact, approximately 60% of total paper usage in the world is for newsprint and office/printing products (Berry 2009).

Chemical pulping:

Traditionally practiced chemical pulping involves treatment of wood with different chemicals to remove noncellulose wood components and leaving intact the cellulose fibers (Smook 1992b, Biermann 1996b). Chemical pulps are made by digesting raw materials, using sulfate (kraft) and sulfite. In the sulfate process wood chips are treated with sodium hydroxide (NaOH) and sodium sulfide (Na_2S) for production of brown stock and then washed with water to remove cooking (Black) liquor for recovery of chemicals and in addition to this sodium sulfate is added in the recovery cycle to compensate for chemical losses as Chemical recovery is an essential part of the pulp production process (Tran 2007, Vakkilainen 2000, Bajpai 2008, Biermann 1996c). It is most dominating and frequently used pulping procedure around the world is also known as the sulphate (Kraft) process (Damiano *et al* 2003).

Sulfite process uses different chemicals to remove lignin. The sulphite process can be carried out only with highly alkaline cooking liquor (Smook 1992b, Biermann 1996b). However, in both kraft and sulphite chemical pulp processing removal of the lignin occurs but certain amount of hemicelluloses and cellulose also degraded so the

yield of pulp is low, usually between 40% to 50% of the original wood substance (Hon and Shiraishi 2001). Kraft pulps produce the strongest papers and are preferentially utilized where strength is required, while sulphite pulps are weaker than equivalent kraft sheets (Berry 2009).

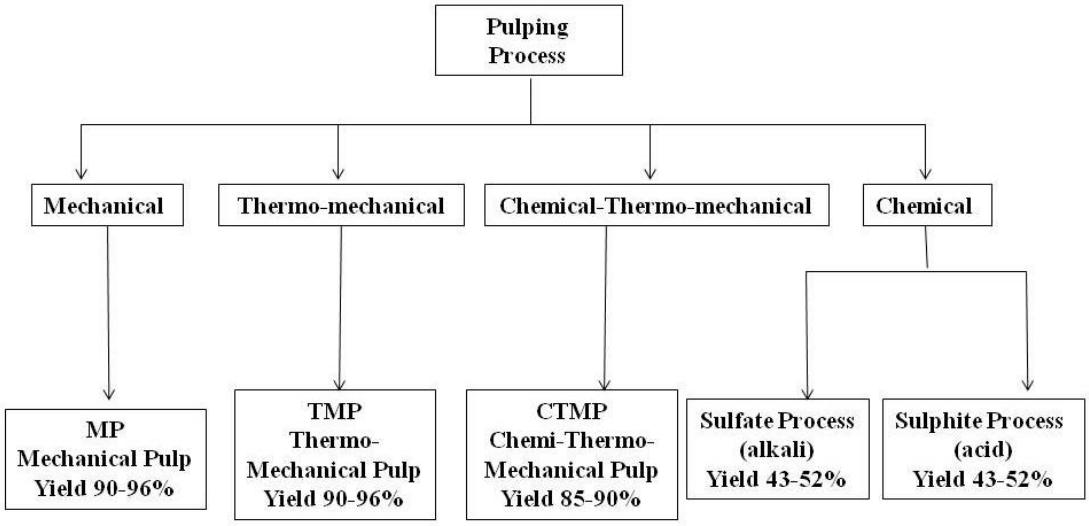


Fig. 1 An overview of pulping process

The major advantage of mechanical pulping over chemical pulping is its high yield of fibers up to 90%, but damage of fibers also taking place as mechanical pulps are mostly produced from softwood because hardwood fibres are severely damaged and yield the finer material that forms a much weaker sheet (Stokke and Groom 2006) and in chemical pulping yield is approximately 50% and strength of fiber is also high (Bajpai 2012). By chemical pulping 95% of lignin is successfully removed while by mechanical pulping considerable fraction of the original lignin content is retained and leads to brightness reversion and coloring (Li, Tan and Yan 2006).

BLEACHING

During the pulp processing, approximately 5-10% of the lignin of the raw materials remains in the pulp which is responsible for the brown colour of the pulp. For the production of bright papers and pulp products, fibres needed further treatment

to remove residual lignin remaining in the pulp after the pulping process and the last step of the pulping process, Bleaching is carried out in which remaining fraction of lignin is being removed and whitening and brightening of the pulp occurs (Pokhrel and Viraraghavan 2004).This process of bleaching (delignification and brightening) uses several chemicals and processes.

The production of white paper by bleaching of pulp includes five or optional six treatment steps with sequentially elemental chlorine (C1), alkali (E1), optional hypochlorite (H) stage, chlorine dioxide (D1), alkali (E2), and chlorine dioxide (D2) (Bahar 2009).

The first two stages primarily release and extract lignin, and the subsequent stages remove the lignin residues and finish the product. These bleaching sequences are applied to maximize the bleaching effect of each component. Water is used to perform intermediate washes to remove extracted waste from the pulp (Gullichsen and Fogelholm 2000, Stenius 2000).

To increase the efficiency of the process for removal of lignin a number of processes and variants of existing processes are available which includes chlorination in which treatment with molecular chlorine is carried out and have been widely used in the industry (Owens 1991). Chlorine has traditionally been used as a bleaching agent because of its strong electrophilic or oxidizing properties but unfortunately because of these characteristics it can also have adverse effects in the environment. During the bleaching process a number of natural substances may become substituted with chlorine. Substitution of organic compounds with chlorine reduces the reactivity of the resulting compound. This stability increases biological environmental persistence and this property further increase with increasing chlorination. The organochlorine compounds produced in the pulp bleaching are of greatest concern

which is persistent, highly bioaccumulative, and toxic. These properties are similar to those of other persistent organic pollutants such as the chlorinated biphenyls (PCBs), dioxins (PCDDs), dibenzofurans (PCDFs), chlorinated benzenes, and pesticides such as DDT (Ritter and Solomon 1995) and represent a relatively rare combination of characteristics that result in higher risks to the environment.

In the first stage of bleaching chlorine acts as oxidizing agent and about half of the chlorine applied to pulp combines with the lignin and the remainder oxidizes the lignin and converted to chloride ion. After alkaline extraction about 90% of the original chlorine applied to pulp is converted to chloride ion but 10% remains as chlorinated organic compounds but dichlorinated products produced due to reaction with chlorine are stable in subsequent alkali phase. The chlorinated pulp is washed before being sent to the alkali extraction stage.

Alkaline extraction removes the soluble colored components and lignin released in the preceding delignification stage (chlorination or oxygen), therefore reducing the amount of bleaching chemicals needed in subsequent stages and improving the durability of the pulp. After alkaline extraction, 80 to 90 percent of the lignin is removed (Van Lierop *et al* 1987).

Chlorination is sometimes repeated after extraction if additional delignification is needed, but because of possible cellulose damage a chlorine dioxide stage is often used. If molecular chlorine is excluded, the term applied for the bleaching sequence is chlorine dioxide bleaching or elemental chlorine-free bleaching. Chlorine dioxide is used to completely replace chlorine in the first delignification stage (Reeve 1987).

The bleaching process in which chlorine leads to production of the wastewaters contain high organic content, dark brown coloration, adsorbable organic

halide (AOX), toxic pollutants, wood processing residuals. Disposal of such wastes cause environmental problems because of high organic content, partitioning of chlorinated organic and trace amount of heavy metal content (Monte *et al* 2009). Organic compounds produced as a result of chlorine bleaching are of major concern, conventional bleaching practices rapidly replaced by process known as Elemental chlorine free bleaching (ECF) in which chlorine is completely substituted by chlorine dioxide which helps in reducing amount of adsorbable organic halide (AOX) (Pryke 2003, Sixta 2006).

Chlorine dioxide is highly selective for lignin, thus reacts rapidly with lignin without affecting cellulose much. When chlorine dioxide reacts with lignin itself reduces to chlorite ion and hypochlorous acid. Chlorite ions decompose to reform chlorine dioxide and chloride ions or they react with the hypochlorous acid to form chlorate ions (Ni *et al* 1992). Hypochlorous acid may react with organic material to form chlorinated organic compounds chlorine dioxide, result in reductions in the quantities of organochlorines produced leads to reduced persistence, reduced potential for bioaccumulation and food chain transfer, reduced toxicity and reductions in adverse ecological effects (Solomon 1995).

However chlorine dioxide reduces the amount of AOX produced it proves to be very expensive and require high investment (Bisaria *et al* 2003). Increasing concern about the environmental impact of bleaching processes with chlorine and chlorine-based compounds have provided motivation for use of totally chlorine free (TCF) bleaching sequences (Shatalov and Pereira 2005, Abrantes *et al* 2007). Totally chlorine free (TCF) bleaching processes have been introduced, largely in response to environmental restrictions and market demands for non-chlorine based chemicals bleached pulps (Dence and Reeve 1996). The bleaching process which is not using

elemental chlorine or its compounds is known as Total chlorine free bleaching. TCF sequence only uses oxygen-based chemicals, such as molecular oxygen, ozone, Hydrogen peroxide etc.

Hydrogen peroxide is mainly used as oxidative bleaching agent to bleach secondary fibers. Hydrogen peroxide produces perhydroperoxyl through ionization reaction which generally act actively in alkalaine condition. However in presence of metal ions perhydroxyl ions are decomposed in the oxygen and water which is responsible for decreasing bleach ability therefore different additives need to be used. (Gierer *et al* 2001). The hydrogen peroxide bleaching system mainly consists of two chemical agents, hydrogen peroxide and the activator. The alkaline activators generally applied for pulp bleaching and when activator reacts with hydroperoxyl in alkaline solution and peracetic acid is produced, which has a stronger bleaching ability (Zhao *et al* 2003).

Another modified lesser polluting delignification process is the oxygen delignification process. Oxygen delignification is a process which uses oxygen and alkali to remove substantial fraction of lignin that remains after pulping. The oxygen delignification process is operated at relatively high temperature and pressure single or two-stage system. Pulp generated after cooking process is washed and mixed with alkai and oxygen. The degree of delignification achieved is normally in the range of 40% to 60%. But the chemicals used to generate alkaline conditions should be compatible with cooking chemicals so mainly Magnesium bisulfite (MgO) is used and only few mills have applied this bleaching process (Bajpai 2011).Oxygen delignification is used to reduce kappa number in bleaching process leads to lower AOX and lower bleaching cost but still require high investment so even if an attractive process but because of economical problems it becomes inefficient.

In the bleaching process efficiency of ozone is widely known and ozone is more efficient delignification agent than oxygen (Leporini *et al* 2004). Ozone is used to reduce chemical cost and it is used just after oxygen delignification stage to finalise removal of lignin before final bleaching process and ozone is not only used as delignification agent but also as brightness booster (Chirat *et al* 2005).

During the kraft pulping process xylan is redeposited on the fibers that creates barrier in the removal of lignin from the pulp in subsequent bleaching process. Treatment of the pulp with xylanase enzyme can remove the xylan barrier and allows better assebility to the bleaching chemicals to remove lignin (Kantelinen *et al* 1993).The use of identified xylanase enzyme helps in the use of chemicals in the bleaching and results in lowering toxicity (AOX) in the effluents (Wang *et al* 1997). Xylanase enzymes used for bleaching of pulps are being developed by many companies which claims their enzymes as suitable bleaching agents. Commercial xylanses like Novozyme 473, VAI xylanase and caratazyme HS 10 were used for prebleaching of kraft pulp which results in 31% chlorine consumption (Bajpai 1994). However enzymes are available in the market but they are highly sensitive to the conditions like temperature, pH and the amount of enzyme to be used for the particular pulp produced by in the industry and proved to be very costly also which affects cost of production. Therefore, it becomes difficult for pulp and paper industry to use the enzyme in the bleaching stage to achieve desired effect.

In order to minimize waste production from pulp and paper mills is the application of best available techniques in which many industries have developed and applied new technologies instead of conventional pulping and bleaching processes like Organic Solvent Pulping which is also known as organosolv pulping. In this process, organic solvent like ethanol, methanol, etc. are preferred. However, this

process is more energy consumer than conventional ones (Sumathi and Hung 2006). This is more economical for small and medium scale plants as significant recovery and reuse of chemicals can also possible. Another widely used method is acid Pulping in which acetic acid under the high pressure is used for treating of wood chips. The disadvantage of this process is to loss of acid, however recovery is possible (Sumathi and Hung 2006).

The major drawback is the waste and wastewaters which are generated from both of pulping and bleaching processes which contain high concentration of chemicals like sodium hydroxide, sodium carbonate, sodium sulfide, bisulfites, elemental chlorine or chlorine dioxide, calcium oxide, hydrochloric acid, etc (Sumathi and Hung 2006) and additional problem is 100 million kg of toxic pollutants are released every year from this industry (Cheremisinoff and Rosenfeld 2010) therefore, an alternative method needs to be worked out.

The most suitable process appears to have the potential to overcome some problems associated with conventional chemical and mechanical pulping methods is Bio pulping which is considered to be effective biological methods as promising alternative to the alkali and chemical bleaches and reduces the utilization of chemicals and energy, pollutants and increase the yield and strength of pulp. (Keller *et al* 2003). The aim of bio pulping process is to eliminate lignin from the wood without loss of cellulose fibers. The process of biopulping was developed to improve the quality of mechanical pulps and significantly reduce the electrical energy required for pulping wood chips (Shukla *et al* 2004, Akhtar *et al* 1998).

BIOPULPING

Biopulping is the process of pre treatment of wood chips and other lingocellulosic materials with natural wood decayed microorganisms prior to thermo

mechanical pulping. In the process of bio pulping wood is debarked, chipped and screened according to normal mill operations. Then chips are briefly steamed to reduce natural chip microorganisms, cooled with forced air, and inoculated with the biopulping organisms. The inoculated chips are piled and ventilated with filtered and humidified air for 1 to 4 weeks prior to processing (Shukla *et al* 2004). This process appears to have the potential to overcome some problems associated with conventional chemical and mechanical pulping methods.

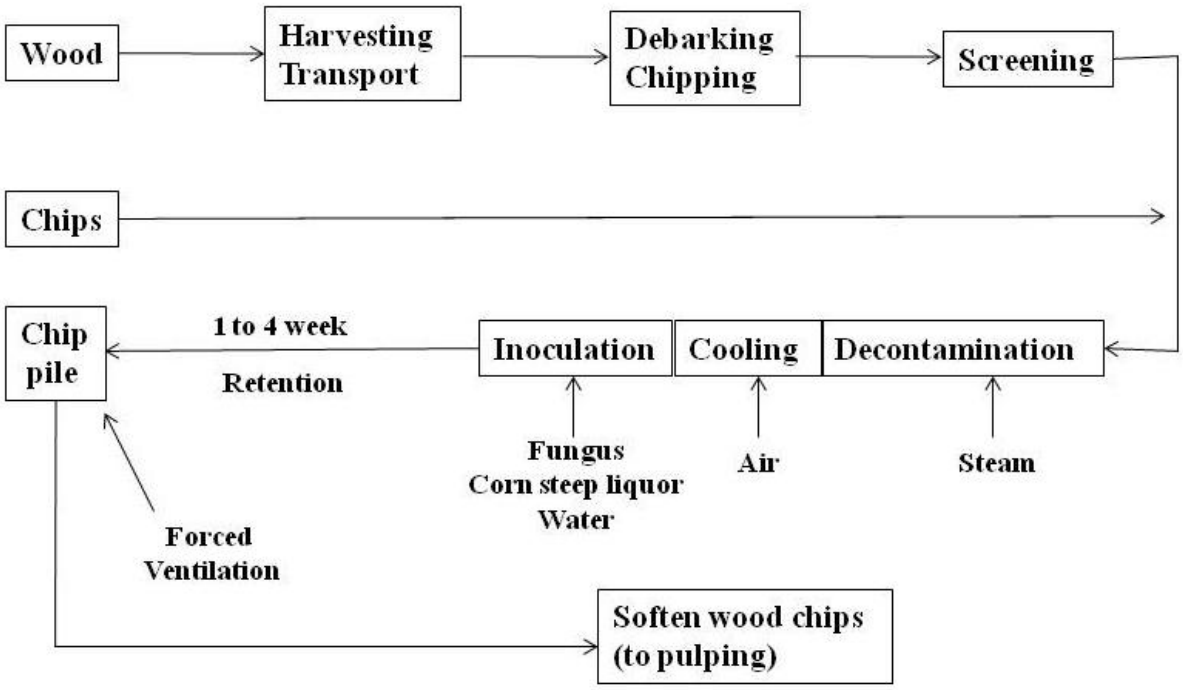


Fig. 2 Overview of the steps involved in biopulping process (Scott *et al* 1998)

Figure 2 represents an overview of the biopulping process suggested by Scott *et al* 1998. Biopulping is an environmentally friendly technology that substantially increases mill throughput or reduces electrical energy consumption at the same throughput in conjunction with mechanical pulping. Electrical energy is the major cost of conventional mechanical pulping. By producing stronger pulp with longer fibers and increased fibrillation, biomechanical pulping may reduce the amount of

kraft pulp required to increase pulp strength. Some selected lignin-degrading fungi can alter cell walls of wood in a short period after inoculation (Bajpai 2012).

A comprehensive evaluation of biopulping at the Forest Products Laboratory (FPL) showed that these fungi can be economically grown on wood chips in an outdoor chip pile-based system. Results also demonstrate the great potential of fungal pretreatment of wood chips prior to chemical pulp production. The most prominent benefit of fungal pretreatment is improved effects on cooking, leading to reduced kappa numbers/reduced active alkali charge and/or reduced cooking time after only 1–2 weeks of fungal treatment. Fungal pretreatment also reduces the pitch content in the wood chips and improves the pulp quality in terms of brightness, strength, and bleachability. The bleached bio pulps are easier to refine than the reference pulps. The process has been scaled up toward industrial level, with optimization of various process steps and evaluation of economic feasibility (Bajpai 2012).

ORGANISMS USED IN BIO PULPING

Many microorganisms, including fungi and bacteria, have been found to be capable of degrading plant cell wall fibers (Mandels and Sternberg 1976).

Bacteria

Certain bacteria may contain the ability to degrade lignified cell walls of wood. Filamentous bacteria belonging to the genus *Streptomyces* are well-known for the degradation of lignin as they solubilize part of lignin, and gives end product water-soluble, acid-precipitable polymeric lignin (Crawford *et al* 1983) and also have capacity to mineralize lignin up to 15% (Crawford and Sutherland 1980, Vicuña 1988, Zimmerman 1990, Godden *et al* 1992, Berrocal *et al* 1997). *Streptomycete* sp.have been shown to degrade low levels of lignin (Crawford 1977, Watanabe *et al* 2003), and multicopper oxidases with laccase activity have been isolated from bacteria,

although these enzymes are expected to mainly participate in sporulation (Claus 2004, Malherbe and Cloete 2002). Eubacteria like *Pseudomonas* spp. are considered to be the most efficient degraders (Vicuæa 1988, Zimmermann 1990). *Actinomycetes* also known to produce extracellular peroxidases (Pasti *et al* 1991, Mercer *et al* 1996) e.g., lignin peroxidase-type enzyme (Ramachandra *et al* 1988, Adhi *et al* 1989).

Nonfilamentous bacteria usually mineralize less than 10% lignin and can degrade only the low-molecular weight part of lignin as well as degradation products of lignin (Rüttimann *et al* 1991, Vicuæa *et al* 1993). Accordingly, bacteria are generally considered secondary lignocellulose degraders, and can degrade cellulose and hemicellulose both aerobically and anaerobically (Walker and Wilson 1991). Examples of aerobic (hemi) cellulose degraders include *Thermobifida fusca* and C*ellulomonas composti*, as well as several other bacteria (Béguin and Aubert 1994). In these cases, degradation is initiated by the concerted activity of cell-associated and free extracellular cellulases and hemicellulases (Walker and Wilson 1991).

Fungi

Lignocellulolytic enzymes-producing fungi are also widespread, and include species from the *ascomycetes* (e.g. *Trichoderma reesei*) and *basidiomycetes* phyla such as white-rot (e.g. *Phanerochaete chrysosporium)* and brown-rot fungi (e.g. *Fomitopsis palustris*). In addition, a few anaerobic species (e.g. *Orpinomyces sp.*) are found to be able to degrade cellulose in the gastrointestinal tracts of ruminant animals (Ljungdahl 2008, Yoon 2007). However, the ability of bacteria to take-up large molecules into the cell is limited and they do not produce extracellular oxidoreductases, they are unable to attack polymeric lignin.

Treatment of wood chips with lignin-degrading fungi prior to pulping has been shown to have great potential for improvements in mechanical and chemical pulping

(Breen and Singleton 1999). The largest group of fungi that degrades wood is the *Basidiomycetes*. Depend up on type of fungal decay Lignin degrading fungi are usually separated into three main groups, causing white, soft, or brown rot (Hataka 2007, Martínez *et al* 2005). This classification is based on the properties and colors of the residual wood. In the case of brown-rot, the brownish colored lignin remains after decay. White rot is characterized by the white-colored cellulose that remains after decay. Soft-rot decay is characterized by surface softness of the wood.

Both brown and white-rot fungi are *Basidiomycetes* that are able to overcome low nitrogen conditions, toxins and antibiotics present in wood. Soft-rot fungi are *ascomycetes* that are able to degrade wood under extreme environmental conditions such as high or low water potential. *Ascomycetes* from genera such as *Daldinia, Hypoxylon,* and *Xylaria* have earlier often been regarded as white-rot fungi, but nowadays these fungi are grouped to soft-rot fungi since they cause typical soft rot. (Nilsson *et al* 1989).

Soft-rot

Soft-rot is caused by a very small group of fungi that mainly attacks hardwoods rendering them soft and crumbly (Rayner and Boddy 1988, Schwarze *et al* 2000). Fungal species associated with this kind of wood degradation may vary in their effects on the cell wall, while sharing features of both white and brown-rot fungi. Soft-rot fungi attack the carbohydrates present in the cell wall, but they also contain oxidative enzyme systems (Rayner and Boddy 1988).

Figure 3 shows shallow depth of decay wood caused by soft rot fungi. Soft-rot fungi are characterized by their preferential growth within the secondary cell wall which is rich in cellulose and they form a number of cavities with conically shaped ends.

Fig. 3 Shallow depth of decayed wood by soft rot fungi (SRF) (Schwarze *et al* 2000)

Figure 4 given below indicated schematic illustration of soft rot fungi. Some soft-rot fungi are able to degrade cellulose using endo-1, 4-β-glucanases, exo-1, 4-β-glucanases and 1, 4-β-glucanases (Schwarze *et al* 2000). Other species do not utilize exo-1, 4-β-glucanases and only degrade the amorphous cellulose zones in the microfibrils.

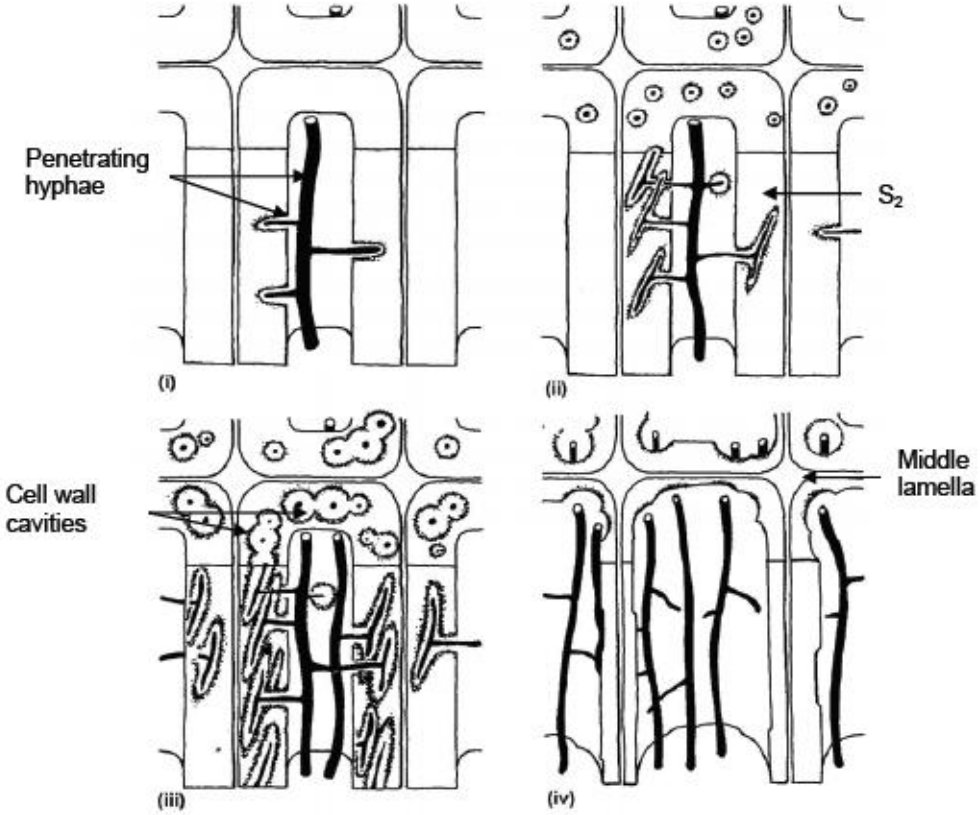


Fig. 4 Different stages of-soft rot (Adapted from Schwarze *et al* 2000).

(i) The hyphae penetrate the lignified cell wall. (ii) Hyphae form branches parallel to the direction of the cellulose microfibrils in the S2 layer. (iii) Cavities form in the cell wall due to degradation. (iv)Here the secondary wall is almost completely degraded, while the compound middle lamella stays intact.

Chaetomium, *ceratocystis* and *Kretzschmaria deusta* are well known to cause soft rot. *Ascomycetes* and *deuteromycetes* generally cause soft-rot decay of wood i.e. when the wood is decayed it has brown, soft appearance (Blanchette 1995, Daniel and Nilsson 1998).

Brown-rot

Brown-rot fungi represent approximately about 6% of the known wood-rotting Basidiomycetes and grow mainly on softwoods (Schwarze *et al* 2000). As the hyphae of fungi penetrates it starts to degrade carbohydrates present in the cell wall by keeping some distance from the hyphae by a diffusion mechanism leaving a modified residue of lignin. This diffusion mechanism is based on the ability of fungi to secrete hydrolases that use cellulose and hemicellulose as substrate (Zabel and Morrell, 1992).

Figure 5 shows brown appearance and cubical checking in the wood decayed by Brown rot fungi and Figure 6 indicated a schematic illustration of brown rot fungi.

Fig. 5 Cubical checking and brown appearance of wood degrading by BRF (Schwarze *et al* 2000).

Brown rot infected wood rapidly loses its inherent strength and undergoes drastic shrinkage and cracking across the decayed wood fibers. In the advanced stages the wood remains as cubic, dark brown crumbly chunks composed mainly of modified structures of lignin (Goodell *et al* 1997).

The different stages of brown-rot caused with the help of synergistic action of a number of fungal enzymes. By the effect of glucose oxidase, glyoxal oxidase, and aryl alcohol oxidase (Evans and Hedger 2001). Hydrogen peroxide is formed and penetrates the cell wall and depolymerizes the lignocelluloses matrix (Schwarze *et al* 2000, Rayner and Boddy 1988).

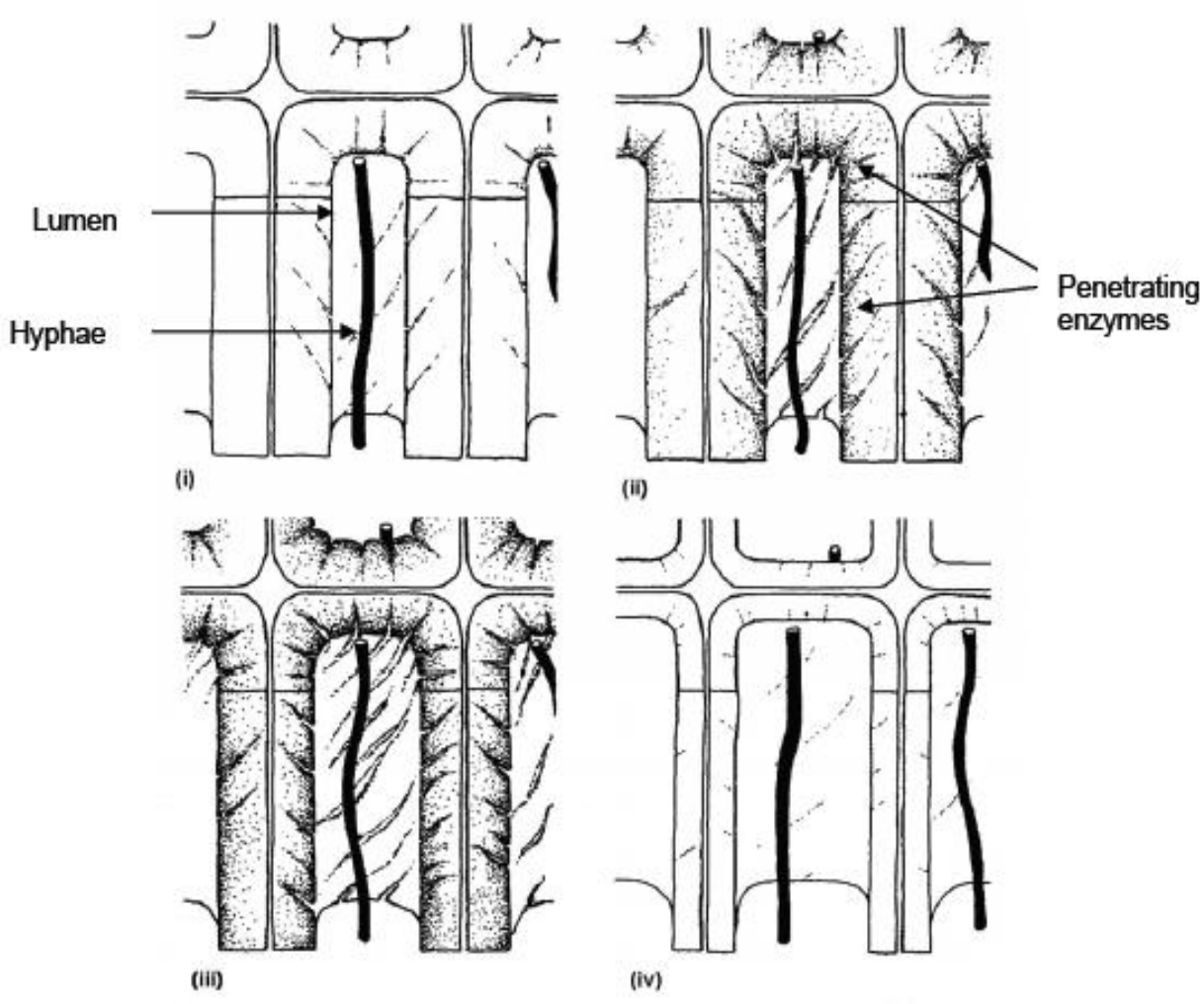


Fig. 6 Different stages of brown-rot (Adapted from Schwarze *et al* 2000).

(i) The enzymes start to penetrate the cell wall from the lumen. (ii) The degree of degradation starts to increase as enzymes penetrated the secondary wall. (iii) Cracks appear in the cell wall and the volume of the latter starts to decrease. (iv)Only modified lignin remains at this stage of the degradation.

This makes cellulose and hemicellulose more accessible to fungal hydrolases. Once the separation of the cellulose chains occurred, endo-1, 4-β-glucanases cleave the cellulose molecule and 1,4-β-glucosidases transform the cellobiose to glucose. Due to the rapid depolymerization of carbohydrates, the water solubility of the lignocellulose may also increase during this stage of the degradation process. This type of wood depolymerization occurs more rapidly than the resulting degradation products metabolization. Consequently, the partially degraded lignocellulosic material and smaller degradation products become available to scavenger fungi and bacteria on the wood. The final product of the decayed wood is brown, dry, brittle and powdery. This residue predominantly composes of modified lignin (Rayner and Boddy 1988). Brown-rot fungi mainly decompose the cellulose and hemicellulose components in wood, but they can also modify the lignin to a limited extent (Eriksson *et al* 1990). The brown color indicates the presence of modified lignin in wood. Many brown-rot fungi such as *Serpula lacrymans*, *Coniophora puteana*, *Meruliporia incrassata*, and *Gloeophyllum trabeum* are destructive to wood used in buildings and other structures (Blanchette 1995).

White-rot

The role of white-rot fungi in forest ecosystems is of great importance (Otjen and Blanchette 1986, Myneni *et al* 2001). They are the only fungal group that contains ability of degrading all three chemical constituents of wood, namely cellulose, hemicellulose and lignin (Rayner and Boddy 1988, Schwarze *et al* 2000) and resulting

in this type of rot. Figure 7 showed bleaching of wood degraded by white rot fungi. Typically white rot appears as a spongy, stringy, or laminated structure in affected wood, where lignin and polysaccharides present in sound wood are removed in equal proportions, although lignin removal may be preferred (Schwarze 1995).

Fig. 7 Bleached appearance of wood due to decay caused by White rot fungi

(Schwarze *et al* 2000)

White-rot fungi secrete hydrolases that acts on cellulose and hemicellulose, while lignin degradation requires more complex enzymes such as lignin peroxidase (Lip), manganese peroxidase (MnP), and laccase (Zabel and Morrell 1992, Leonowicz *et al* 1999). Ligninolytic fungi produce monosaccharides from polysaccharide components in wood by using hydrolases to (Leonowicz *et al* 1999). However, when these components are in a complex with lignin, hydrolytic breakdown does not occur. Thus, lignin appears to inhibit hydrolytic activity (Martínez *et al* 2005).

The only organisms capable of mineralizing lignin efficiently are *Basidiomycetes* white rot fungi and related litter-decomposing fungi (Kirk and Cullen 1998). Most white rot species have the ability to disintegrate and mineralize lignin (Hunt *et al* 2002). It is known that the biopulping effect is caused by the lignin-degrading system of white-rot fungi. There has been quite little correlation between removal of specific components of the wood by the fungi and efficacy of the fungal pretreatment in either energy savings or paper strength property improvement (Bajpai

2012). White rot fungi and their enzymes especially ligninases and xylanases are considered for the wood chips treatment of prior to pulping. While ligninases attack the lignin content of wood, xylanases degrade hemicelluloses and make the pulp more permeable for the removal of residual lignin. Thus, biopulping process not only removes lignin but also some of the wood extractives, thus reducing the pitch content and effluent toxicity (Ali and Sreekrishnan 2001).

Various species of white rot fungi have been used for biopulping, however, *Ceriporiopsis subvermispora* has proven to be very competitive both on softwoods and hardwoods (Ferraz *et al* 2007). The physiology and biochemistry of *C. subvermispora* has been studied to allow an intensification of the biopulping process (Sethuraman *et al* 1998, Milagres *et al* 2005).

It was also reported that *Pycnoporus sanguineus* was able to reduce lignin content by 11% in 14 days of treatment, but *P. taeda* wood also suffered a notable structural changes in lignin and hemicelluloses during the treatment (Levin *et al* 2007). Bajpai *et al* (2001, 2003) observed that extractive content reduced by 17–39% when *eucalyptus* chips treated with the fungus *C. subvermispora* for 2 weeks and were subjected to Kraft pulping Brightness and strength properties of biopulps were better than the control and the pulps were easier to bleach and easier to refine requiring less energy (by 18–30%).

Garmaroody *et al* (2011) has done the research in which poplar chips were pretreated by *Trametes versicolor* for 1, 2, and 3 weeks and then after washing, the chips were air-dried for kraft pulping to achieve pulp kappa number of around 20. Analysis of the pulp samples indicated that fungi pretreatment of chips can degrade lignin and carbohydrates and affect kraft pulping and fiber characteristics.

Bajpai *et al* (2004) evaluated the effect of pretreatment of wheat straw with

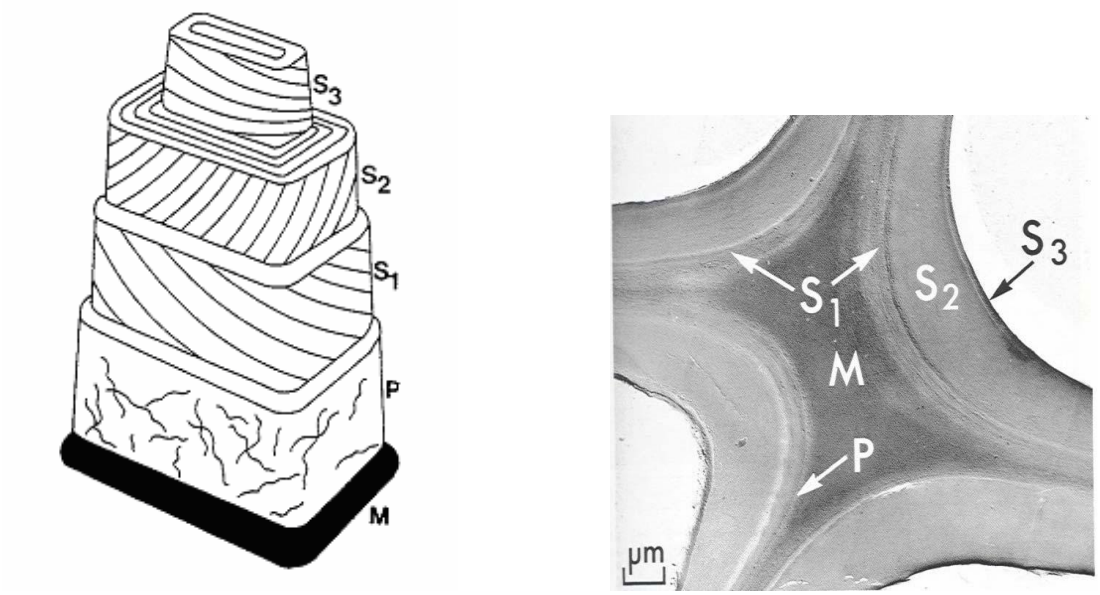
lignin-degrading fungi on chemical pulping. Treatment with *C. subvermispora* reduced the lignin and extractive content of wheat straw by 16.5 and 44.3%, respectively. Bajpai *et al* (2004) also studied the pretreatment of bagasse with *C. subvermispora* strains and its effect on chemical pulping. Treatment of depithed bagasse with different strains of *C. subvermispora* reduced the kappa number by 10–15% and increased unbleached pulp brightness. Yaghoubi *et al* (2008) used *C. subvermispora* for biochemical pulping of agricultural residues and the results were compared with chemical pulping. Biological treatment of rice, wheat and barley straw samples resulted in decrease of the kappa number by 34, 21 and 19%, respectively, as compared with controlled samples (Yaghoubi *et al* 2008).

Fungi are microorganisms which live on and within wood and slowly digest the cell wall materials leading to softening and decay. Wood degrading fungi have general requirements for survival like moisture, oxygen, nutrients, favorable temperature, suitable pH and a nontoxic substrate (Blanchette and Hoffman 1994, Eaton and Hale 1993).Wood decay fungi obtain nourishment by digesting cell walls, and causing deterioration of wood. In order to break down the chemical components of the cell wall in the wood fungi produce enzymes capable of attacking these components (Vmezurike 1968). Enzymes are secreted at the tips of hyphae which while its penetration through the wall attack the cell wall components like lignin, cellulose and hemicelluloses and break them into simple sugars making it available for its nutrition. The detailed structure of the plant cell was is described below.

STRUCTURE OF THE PLANT CELL WALL

The typical lignified cell wall consists of five cell-wall layers as shown in the figure 8 A and B bellow. On the outer side the middle lamella (M) is present, the primary wall (P), and a three layers of secondary wall consisting of the outer (S_1), middle (S_2) and

inner (S_3) secondary cell wall layers on the inner side (Schwarze *et al* 2000). These layers differ in their structure, chemical composition and orientation of the microfibrils.



(a) (b)

Fig. 8 Model of cell wall layers (Schwarze *et al* 2000, Sjöström1993)

Figure 8 (a) represents a model of cell wall five different cell wall layers (Schwarze *et al* 2000) and 8 (b) shows a transmission electron micrograph of early wood tracheids showing the different layers of the cell wall. Scalebar = 1 µm. (Sjöström1993).

Middle lamella

The middle lamella layer consists mainly of amorphous substances like pectin and lignin (Schwarze *et al* 2000). This layer connects neighboring cells to allow the movement of biochemicals and water (Wiedenhoeft and Miller 2005). Pectin acts as a cement-like substance for cell elements in non-woody organs, while lignin provides rigidness in the wood cell.

Primary wall

In general, the primary wall in wood is thin and indistinguishable from the middle lamella (Schwarze *et al* 2000, Wiedenhoeft and Miller 2005).Thus, These two layers

are also known as the compound middle lamella. The primary wall consists of randomly orientated cellulose microfibrils embedded in a highly hydrated matrix providing strength and flexibility to this layer. Cell wall matrix consists of two major groups of polysaccharides hemicellulose and pectin. It also consists of small amount of structural protein.

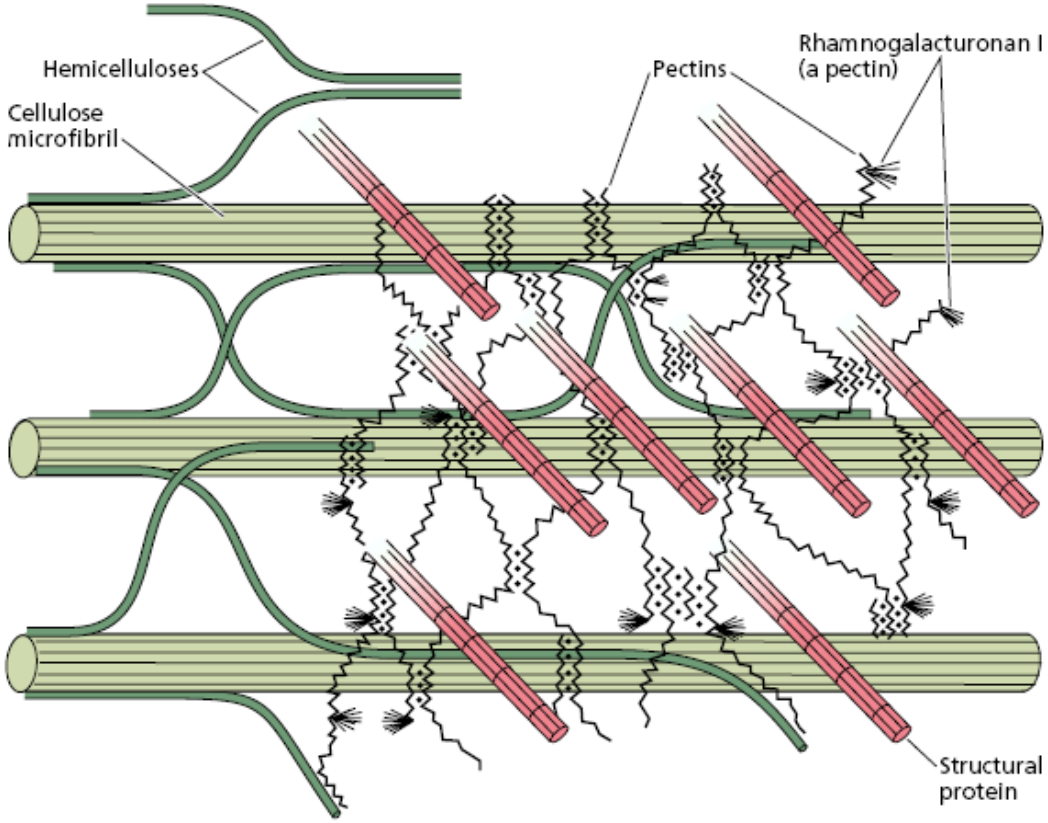


Fig. 8 (C) Schematic diagram of the major structural components of the primary cell wall and their likely arrangement (From Brett and Waldron 1996.)

Figure 8 (C) represented cellulose microfibrils are coated with hemicelluloses like xyloglucan, which also cross-link the microfibrils to one another. Pectins form an interlocking matrix gel, perhaps interacting with structural proteins.

Secondary wall

This three layer cell wall comprising of 94% cellulose, represents the largest part of the cell wall (Schwarze *et al* 2000, Wiedenhoeft and Miller 2005). The primary function of this layer is to provide strength to the cell.

The outer secondary wall (S_1) is a thin layer next to the primary wall. The cellulose fibers of this layer show a weak parallel arrangement to the longitudinal axis of the cell (Wiedenhoeft and Miller 2005). The middle secondary wall (S_2) forms the largest part of the secondary wall and played the most important role in establishing the properties of the cell. The fibrils are arranged parallel to each other in a spiral in the direction of the cell's longitudinal axis. This layer has a low lignin and high cellulose content and it was found to be the preferred substrate for brown and soft rot fungi as these two groups can only degrade cellulose (Schwarze *et al* 2000, Wiedenhoeft and Miller 2005). The inner secondary wall (S_3) is a relatively thin layer and separates the cell wall from the lumen (Schwarze *et al* 2000).The arrangement of the microfibrils in this layer resembles those of the primary cell wall. The inner secondary cell wall has the lowest percentage of lignin compared to the other layers of the secondary wall (Wiedenhoeft and Miller 2005).

COMPONENTS OF WOOD

The major components of the wood cell walls are three biopolymers, cellulose, hemicellulose, and lignin (Harris and Stone 2008).

Cellulose

Cellulose is the most abundant and significant polysaccharide on the earth, made up of cellobiose subunits linked by β-1,4 linkage. Wood species contain 40-45% (as dry weight) cellulose (Eriksson *et al* 1990). Cellulose is a long, linear homopolymer as shown in the figure 9 consisting of β-D-glucose residues with (1→4) glucosidic linkages (Zabel and Morrell 1992, Rowell *et al* 2005) and the degree of polymerization is up to about 15000 glucose units in one polymeric chain (Kuhad *et al* 1997). The anhydroglucose monomers on the surface of the cellulose molecules each contain three hydroxyl groups. These groups determine the physical and

chemical properties of the wood, as well as the structural properties in the cell wall. In wood cell wall the long, cellulose chains are stabilized by hydrogen bonds to form microfibrils and further they form cellulose fibers. Cellulose molecules tend to form intra- and intermolecular hydrogen bonds.

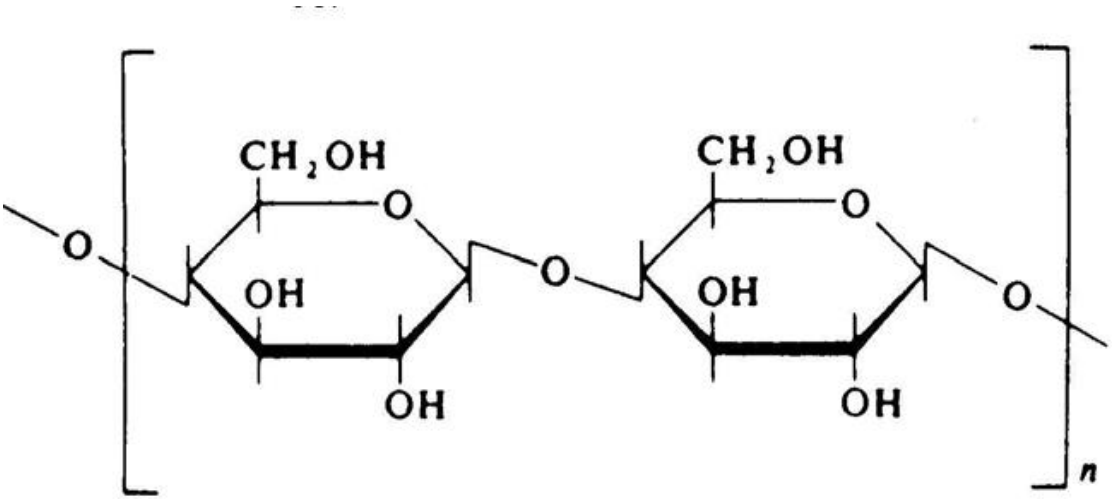


Fig. 9 Structural formula of cellulose (Raymer and boddy 1988)

Crystalline regions are then formed as the packing densities of cellulose increases. As much as 65 % of wood derived cellulose may be crystalline. Cellulose is present in a major amount in thick S_2 layer of xylem element secondary wall where the fibrillous structure gives mechanical strength (Argyropoulos and Menachem 1997).

Highly organized crystalline cellulose is accessible on the surface but not inside the crystal so not easily degradable. Amorphous non organized non crystalline cellulose is mostly accessible, but are covered with hemicellulose and lignin rendering the molecule non-accessible. However non crystalline cellulose is more susceptible to enzymatic degradation (Kuhad *et al* 1997).

Hemicellulose

Hemicellulose plays important role of a supporting material and comprise 20-30% dry weight of wood (Sjöström 1993). Hemicelluloses are more easily biodegradable than

cellulose because they are amorphous and have a moderate degree of polymerization (100-200 units). Hemicellulose differs from cellulose as it consists of a shorter carbohydrate backbone containing other sugar monomers than just glucose, and side chains that can be branched (Rayner and Boddy 1988, Rowell *et al* 2005).

Hemicelluloses are a group of branched heteropolysaccharides consisting of different hexose sugars, pentose sugar, and sugar acid units D-xylopyranose, D-glucopyranose, D galactopyranose, L-arabinofuranose, D-mannopyranose, D-glucopyranosyluronic acid, and D-galactopyranosyluronic acid.

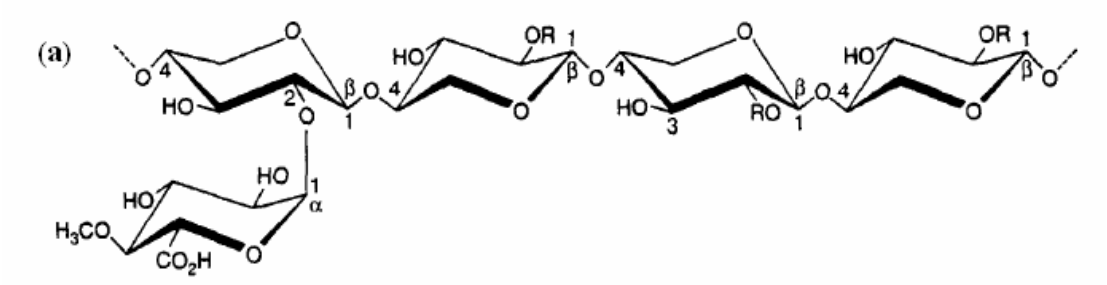


Fig.10 (a) the structure of O-acetyl-4-O-methylglucuronoxylan, the major hemicellulose of hardwoods (Kirk and Cullen 1998).

The composition and structure of hemicelluloses differ in softwood and hardwood. Figure 10 (a) indicated the structure of hemicellulose in hardwoods. In the hard wood hemicelluloses consists of Glucuronoxylan and are characterized by a backbone of D-xylopyranose monomers that are β-(1→4) linked to acetyl groups (Rowell *et al* 2005). In the backbone, side chains of 4-*O*-methylglucoronic acid monomers are linked to the xylan and substitute the xylan with intervals.

In softwood, the hemicellulose consists of glucomannans and has a slightly branched chain with β-(1→4) linkages as represented in figure 10 (b). Another hemicellulose polymer in softwoods is an arabinoglucoronoxylan consisting of a backbone of β-(1→4) xylopyranose units and branches containing *D*-glucopyranosyluronic acid and *L*arabinofuranose (Rowell *et al* 2005).

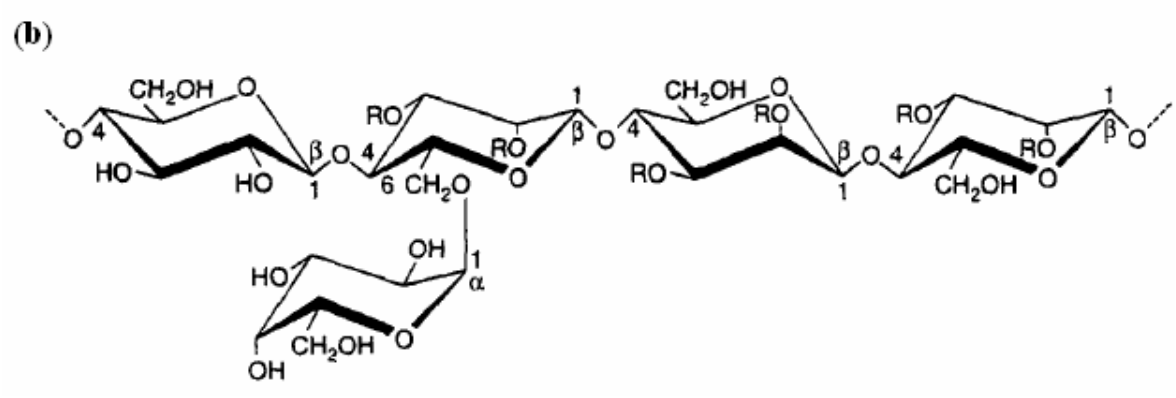


Fig. 10 (b) structure of O-acetylgalactoglucomannan, the major hemicellulose of softwoods (Kirk and Cullen 1998).

Lignin

Lignin is the second most abundant polymer next to cellulose (Boerjan *et al* 2003, Lebo *et al* 2001) occurs in all the vascular plants and comprises 20-30% of the wood cell wall (Zabel and Morrell 1992) which provides strength to the plant and protects the stem tissue. It is composed of phenyl propane units linked together by carboncarbon (C-C) and ether (C-O-C) linkages Lignin is mainly present in the middle lamella region of wood, but most of the lignin is present within the secondary wall.

In the secondary wall lignin is mixed with and covalently bonded to the hemicelluloses and the cellulose fibrils are embedded in the lignin-hemicellulose matrix. 2-5% of the wood dry weight is made up of extractives (Sjöström and Westermark 1998) which are non-structural constituents of wood broadly divided into terpenes, resins, and phenols (Kuhad *et al* 1997). These different organic compounds have different roles like, acting as a nutrition reserve for the living wood cells, and giving protection against microbial degradation (Sjöström 1993).

It consists mainly of dimethoxylated (syringyl), monomethoxylated (guaiacyl), and non methoxylated (*p*-hydroxyphenyl) phenylpropanoid monomers (Zabel and

Morrell 1992, Rowell *et al* 2005). *p* -coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol are the precursors of lignin biosynthesis present in the xylem cells of the plants structure of which are as shown in figure 11a, b, c.

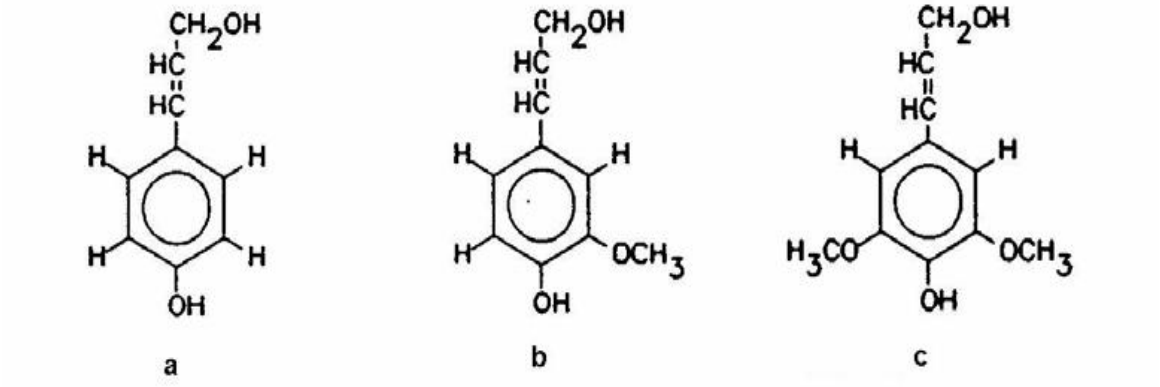


Fig. 11 structures of lignin precursors. (a) *p*-coumaryl alcohol, (b) coniferyl alcohol, (c) sinapyl alcohol (Zabel and Morrell, 1992).

P-coumaryl is a minor precursor of soft- and hardwood lignins, coniferyl is the major precursor of softwood lignin, while coniferyl and sinapyl are both precursors of hardwood lignin. Structural model of lignin suggested by Brunow (2001) is shown in the figure 12.

During lignin biosynthesis due to the action of laccases and peroxidases, these monolignols are polymerized to *p*-hydroxyphenyl (H-type), guaiacyl (G-type), and syringyl (S-type) type of lignin subunits respectively (Higuchi 2006). The composition and amount of lignin varies between softwood and hardwood, and also between plant species. In the softwoods lignin consists mainly of guaiacyl subunits while in hardwood lignin contains both guaiacyl and syringyl subunits (Adler 1977, Higuchi 2006). In grasses, lignin present in cell wall of the xylem element contains *p*-hydroxyphenyl subunits (Eriksson *et al* 1990). Complete chemical structure of plant lignin is still unknown. The structural modifications of native lignin and partial

degradation are considered the main characteristics of this process that facilitate its removal and wood softening in the subsequent pulping processes (Mendonça *et al* 2002).

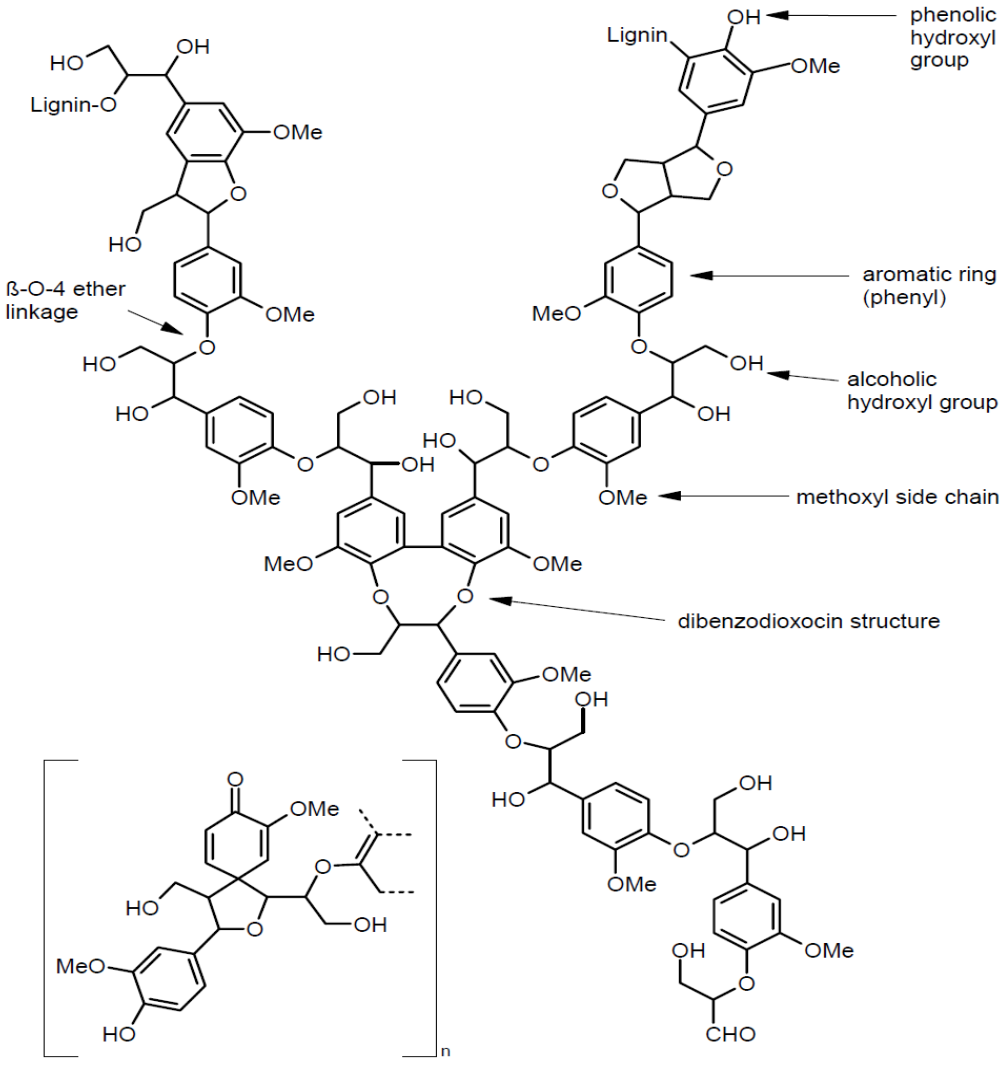


Fig. 12 Structural model of lignin by Brunow (2001)

Due to its complicated structure, lignin is highly resistant to microbial degradation and its association with cellulose and hemicellulose polysaccharides also imparts degradation resistance to these polymers (Hatakka 2001). Several properties of lignin are responsible for its resistance to microbial attack: it is a water-insoluble, aromatic, three-dimensional molecule containing non-hydrolyzable bonds (Brunow 2001). Moreover, the enzymes needed for the complete degradation of lignin are only

induced in the absence of readily available nutrients. Thus degradation of lignin is delayed and only occurs slowly. Two patterns of lignin degradation are known. The fungus can either degrade only lignin selectively leaving cellulose and hemicelluloses unaffected or all three chemical components simultaneously in which carbohydrates are also removed at the same time (Pandey and Pitman 2003, Schwarze *et al* 2000).

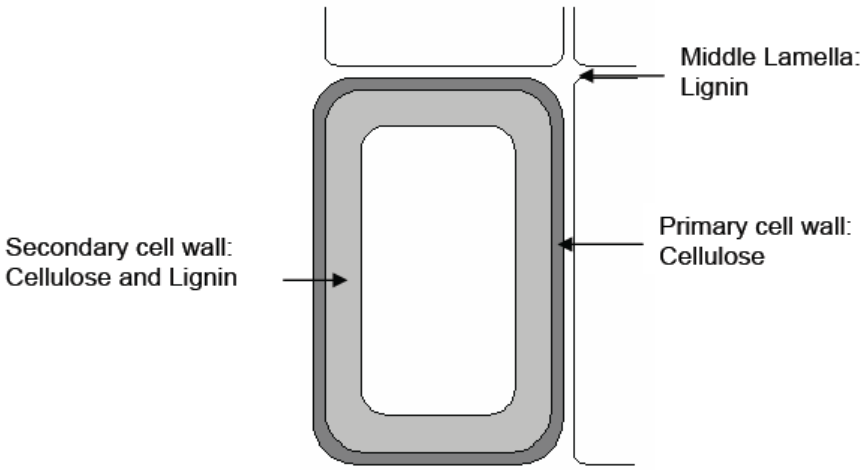


Fig. 13 Chemical components of cellwall

Figure 13 represents the schematic illustration of wood cell wall showing relative position of the main chemical components of the cell wall. Such an illustration is essential to explain the mechanics of fungal degradation of these cells.

In the process of carbon recycling lignocellulose degradation from the cell wall is considered to be a key step (Eriksson *et al* 1990). Basidiomycetes are the main rotters as they are having ability to modify and degrade lignin efficiently. Wood rotting basidiomycetes are classified in to white rot and brown rot depended up on macroscopic appearance (Schwarze *et al* 2000, Zabel and Morrell 1992). White rot basidiomycetes degrades lignin, cellulose, hemicellulose and giving rise white material enriched in cellulose. White rot can degrade lignin selectively or simultaneously with cellulose and two different patterns are characterized namely selective delignification and simultaneous delignification.

Selective delignification

In the process of selective delignification lignin is the first wood component to be degraded. A typical example of this is the wood rot brought about by the fungus *Phellinus pini* (Schwarze *et al* 2000). Schematic illustration of selective delignification caused by white rot fungi is shown in figure 14. Firstly, the middle lamella is degraded together with the secondary wall. Later on the individual cells will become separated from their matrix. This result in loss of its stiffness and compression strength and appearance of wood becomes fibrous and stringy is degraded at a slower rate than in brown or soft rot, and the reduction in wood strength is not as severe as in the brown and soft rot.

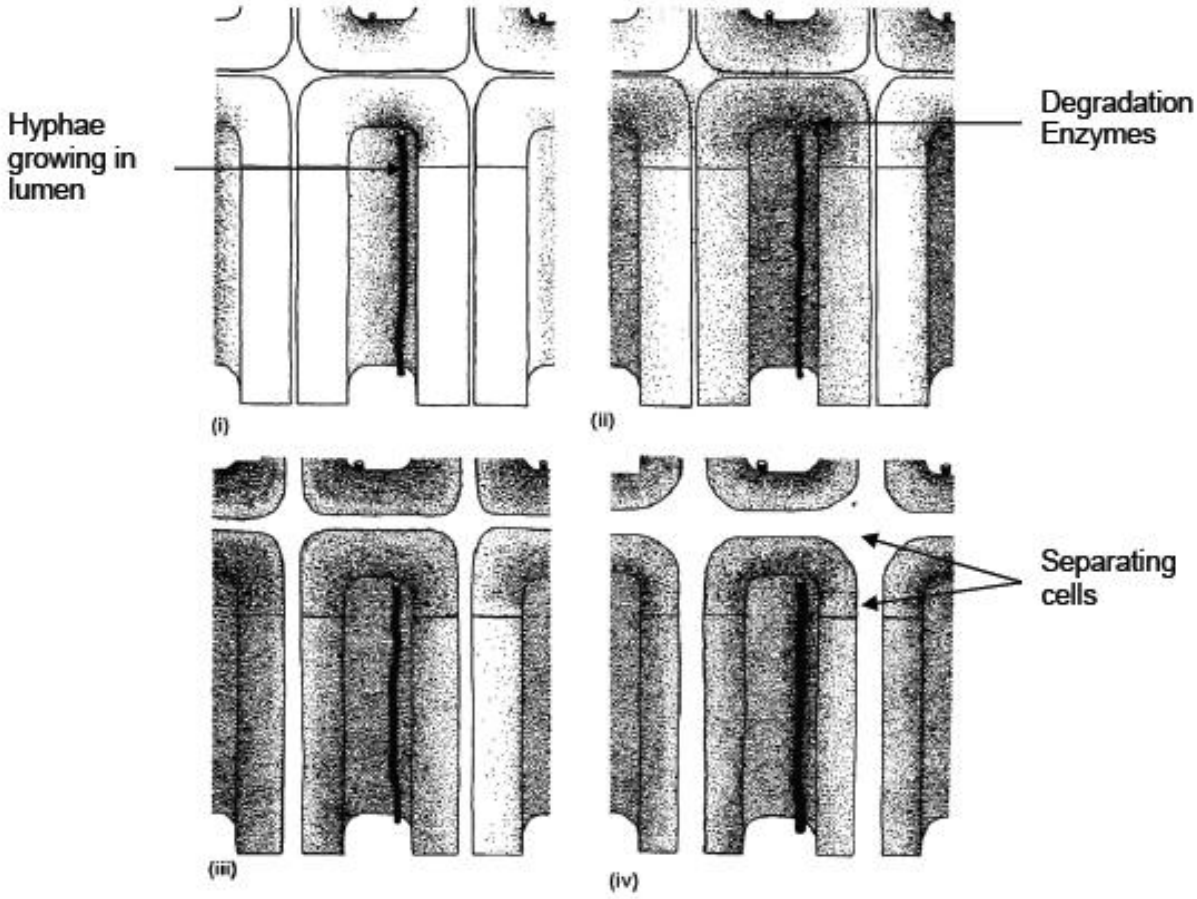


Fig. 14 Stages of selective delignification (Adapted from Schwarze *et al* 2000)

(i) Hyphae grow in the lumen and the degradation enzymes diffuse into the secondary wall where lignin is degraded. (ii) Degradation of secondary wall lignin spreads to the middle lamella. (iii, iv) Later during lignin degradation, the individual cells separate from one another.

Simultaneous delignification

Simultaneous delignification occurs mainly on trees containing broad-leaves where the fungal enzymes are able to degrade all the main components of the lignified cell wall simultaneously. In the simultaneous degradation lignin and polysaccharides were broken down approximately at the same rate (Schwarze *et al* 2000). Figure 15 represents a schematic illustration of simultaneous delignification pattern of white rot fungi. Degradation takes place as the hyphae grows in the lumen and cell wall started to degrade from lumen outwards leads to the formation of erosion channels. The middle lamella and cell corners were also degraded at the final stage of wood degradation.

The degradation of the cell wall is enhanced by a biofilm coating around the hyphae that result in closer contact between the hyphae and the cell wall components (Lynd *et al* 2002). As a result of which the cell wall gradually becomes thinner from the inside as degradation continues. In contrast to selective delignification, the wood in this case becomes brittle because of the degradation of the cellulose-rich secondary wall. Regardless the pattern of lignin degradation in wood, the process of delignification is brought about by the action of the ligninolytic enzymes.

This pattern occurs mainly on hardwoods while selective rot may occur on both hard and softwoods. The selectively lignin degrading fungi is important for the paper and pulp industry, as the residual cellulose fibers are the main component for

making of paper and leads to reduce costs of required chemicals and pollution which occurs during the pulping process (Guitiérrez *et al* 1999).

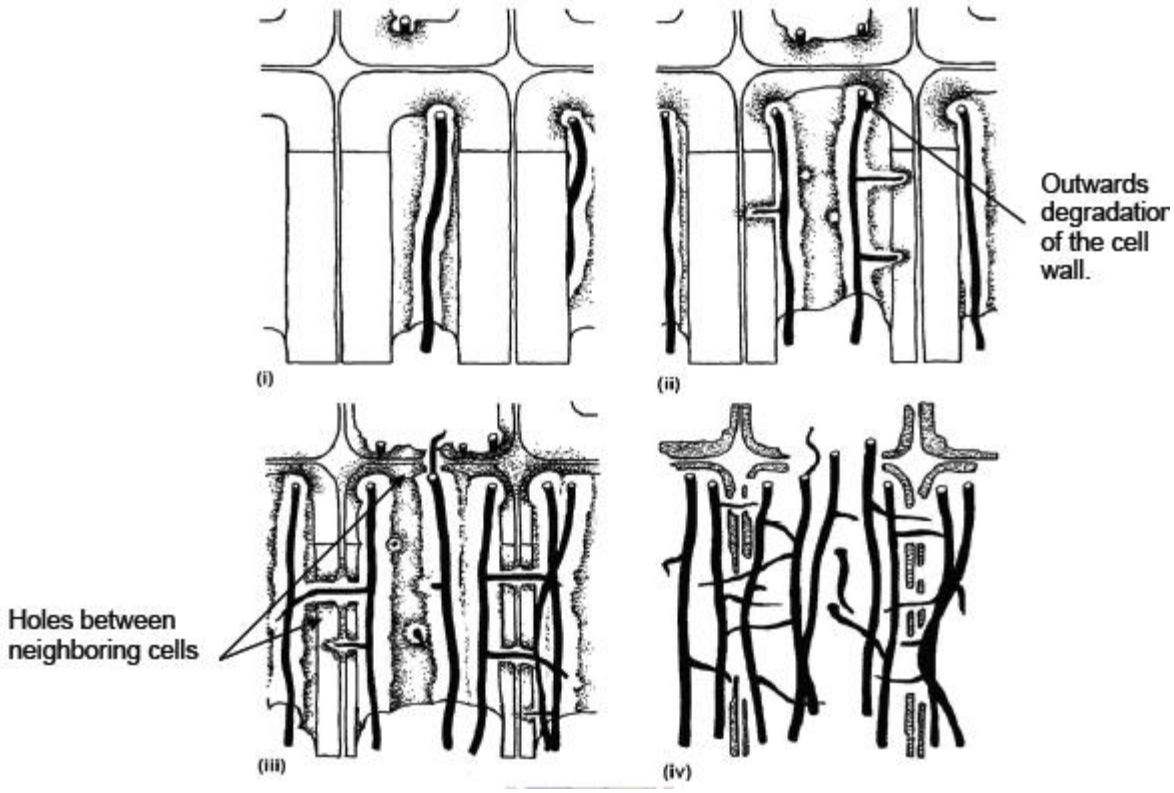


Fig.15 Stages of simultaneous delignification (Adapted from Schwarze *et al* 2000)

(i) The degradation enzymes from the hyphae start to attack the cell wall in their immediate vicinity. (ii) The cell wall is degraded from the lumen outwards. (iii) The cell wall becomes thinner and holes appear between neighboring cells. (iv) At the final stage of degradation, the middle lamella and cell corners are degraded.

Various species of white rot fungi have been used for biopulping, however, *Ceriporiopsis subvermispora* has proven to be very competitive both on softwoods and hardwoods (Ferraz *et al* 2007). Some white-rot fungi preferentially remove lignin without a substantial loss of cellulose, and cause white-pocket or white mottled type of rot, e.g., *Phellinus nigrolimitatus* (Blanchette 1995). There are also fungi that are able to produce both types of attack in the same wood (Eriksson *et al* 1990). Typical

examples of such fungi are *Ganoderma applanatum* and *Heterobasidion annosum*. As fungi selectively degrading lignin are considered the most promising fungi for applications in the pulp and paper industry, the search among these fungi has attained a considerable interest. Degradation/decay by fungi occurred due to specific enzymes (extracellular and intracellular) released by the fungal hyphae.

ENZYMES INVOLVED IN DEGRADATION OF WOOD

Lignocellulose is the major structural component of woody plants and non-woody plants such as grass and represents a major source of renewable organic matter as shown in figure 16. Lignocellulose consists of lignin, hemicellulose and cellulose (Malherbe and Cloete 2003).

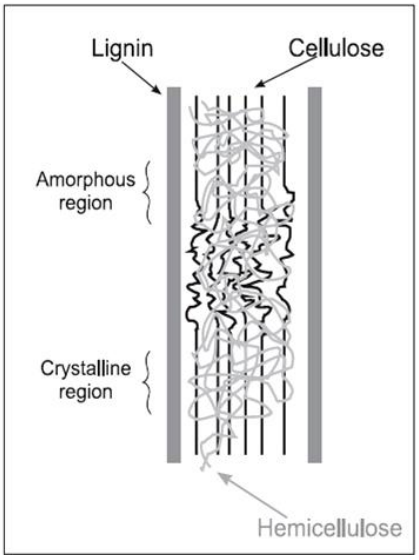


Fig. 16 Structure of lignocellulose (Sjörström 1993)

Due to the complicated structure of lignin it is highly resistant to microbial degradation and its association with cellulose and hemicellulose polysaccharides imparts more degradation resistance to these polymers (Hatakka 2001). It is generally believed that lignin depolymerization is necessary to gain access to cellulose and hemicelluloses (Hatakka 2001). Microorganisms have two types of extracellular enzymatic systems: the hydrolytic system, which produces hydrolases and is

responsible for cellulose and hemicellulose degradation; and a unique oxidative and extracellular ligninolytic system, which depolymerizes lignin (Perez *et al* 2002).

Three main enzymes are important which play major role in the degradation of lignin are lignin peroxidase (LiP), Manganese peroxidase (MnP), and Laccase. These enzymes are described as true ligninases due to having high redox potential (Gold *et al* 2000, Martínez 2002). Other enzymes that are involved in lignin degradation are H2O2 generating oxidases, and mycelium associated dehydrogenases that reduce compounds derived from lignin (Gutiérrez *et al* 1994, Guillén *et al* 1997). Figure 17 showed an illustration of chemical and enzymatic degradation of lignin as suggested by Martínez *et al* 2005.

Lignin peroxidases: (EC 1.11.1.14) were first characterised by Tien and Kirk (1983) as "ligninases", but soon thereafter another ligninolytic peroxidase was found, that required manganese (Tien and Kirk 1984, Kuwahara *et al* 1984). LiPs are heme-containing glycoproteins and play a central role in the biodegradation of the cell wall constituent, lignin (Piontek *et al* 2001). LiP is able to degrade non-phenolic lignin units to aryl cation radicals which then use non enzymatic reactions to cleave C-C and C-O bonds. LiPs catalyze the H2O2-dependent oxidative depolymerization of a variety of non-phenolic lignin compounds and a wide range of phenolic compounds (Wong 2009). LiPs oxidize the substrates in multi-step electron transfers and form intermediate radicals, such as phenoxy radicals these intermediate radicals undergo non-enzymatic reactions such as radical coupling and polymerization, side-chain cleavage, demethylation and intramolecular addition and rearrangement. For degradation of non phenolic compounds it does not require any intermediate mediators due to high redox potential (wong 2009).

Manganese peroxidase: (E.C. 1.11.1.13) are extracellular glycoproteins and are secreted in multiple isoforms which contain one molecule of heme (Asgher *et al* 2008), has a similar catalytic cycle as LiP, but it utilises Mn(II) as a substrate. MnP catalyzes the peroxide dependent oxidation of Mn (II) (as the reducing substrate) to Mn (III). Mn (III) is chelated by organic acids and acts as oxidizer and reacts with phenolic and non phenolic compounds of lignin molecule. MnP is able to liberate CO_2 directly from lignin substructures (Hofrichter *et al* 1999). It can also produce H_2O_2 in O_2-requiring oxidation of NAD(P)H (Glenn *et al* 1986).

Laccases: (E.C. 1.10.3.1) have been studied for over a hundred years. The enzyme is blue copper-containing polyphenol oxidase that uses molecular oxygen to oxidize various aromatic and nonaromatic compounds through a radicalcatalyzed reaction mechanism (Claus 2004 and Baldrian 2006). It catalyses the reduction of molecular oxygen to water. Laccase cause oxidation of the alpha carbon, demethoxylation cleavages in phenyl groups, and Cα – Cβ cleavage in syringyl structures. As the result of lignin decomposition, laccases also provide the quinones and phenoxyradicals that are important in the decomposition of cellobiose through the action of cellobiose dehydrogenase (Zabel and Morrell 1992). The following Figure illustrates the degradation of lignin via enzymatic reactions.

Laccase, LiP, and MnP oxidize the lignin polymer and generate aromatic radicals (a). These radicals may be involved in a number of non-enzymatic reactions including C4-ether breakdown (b), the cleavage of the aromatic ring (c), cleavage of the Cα – Cβ bond (d), and demethoxylation (e). The cleavage of the Cα – Cβ bond in lignin releases aromatic aldehydes that are the substrate for H_2O_2 generation by aryl-alcohol dehydrogenases and aryl alcohol oxidase in cyclic redox reactions. If phenoxy radicals from C4-ether breakdown (b) are not reduced by oxidases to phenolic

compounds (i), they can repolymerize on the lignin polymer (h). Laccase or peroxidases can reoxidize the phenolic compounds formed (j). Phenoxy radicals may also undergo Cα – Cβ breakdown (k), resulting in the formation of *p*-quinones. These quinines indicated by (g) and (k) in figure play a role in oxygen activation in redox cycling reactions (l, m). The ferric iron present in wood is reduced (n) and reoxidized while H2O2 is reduced to a hydroxyl free radical (OH-) (o). The latter is a strong oxidizer and plays an important role in the initial stages of wood degradation, as it attacks the lignin (p) when the pore sizes are still too small for penetration by other ligninolytic enzymes. *Basidiomycetes* white-rot fungi use oxidative enzymes for the degradation of lignin and hydrolytic enzymes to degrade cellulose and hemicellulose.

Cellulases: Cellulase hydrolyze the β-1, 4-glycosidic linkages of cellulose. Traditionally, they are divided into two classes referred to as endoglucanases and cellobiohydrolases. Endoglucanases (endo-1,4- β -glucanases, EGs) can hydrolyze internal bonds (preferably in cellulose amorphous regions) releasing new terminal ends. Cellobiohydrolases (exo-1,4-b-glucanases, CBHs) act on the existing or endoglucanase-generated chain ends. Both enzymes can degrade amorphous cellulose but, with some exceptions, CBHs are the only enzymes that efficiently degrade crystalline cellulose.

Xylanases: These are fast becoming a major group of industrial enzymes finding significant application in paper and pulp industry. Xylanases are of great importance to pulp and paper industries as the hydrolysis of xylan facilitates release of lignin from paper pulp and reduces the level of usage of chlorine as the bleaching agent (Shoham *et al* 1992). Viikari *et al* 1986 were the first to demonstrate that xylanases are applicable for delignification in bleaching process. The complex structure of xylan needs different enzymes for its complete hydrolysis. Endo-1, 4-b- xylanases (1, 4-b-

D-xylanxylanohydrolase, E.C.3.2.1.8) depolymerise xylan by the random hydrolysis of xylan backbone and 1, 4-b-D-xylosidases (1, 4, b-D-xylan xylohydrolase E.C.3.2.1.37) split off small oligosaccharides. The side groups present in xylan are liberated by a-L arabinofuranosidase, a-Dglucuronidase, galactosidase and acetyl xylan esterase (Collins *et al* 2005). These enzymes are required for many applications such as bleaching of Kraft pulp, increasing the brightness of pulp.

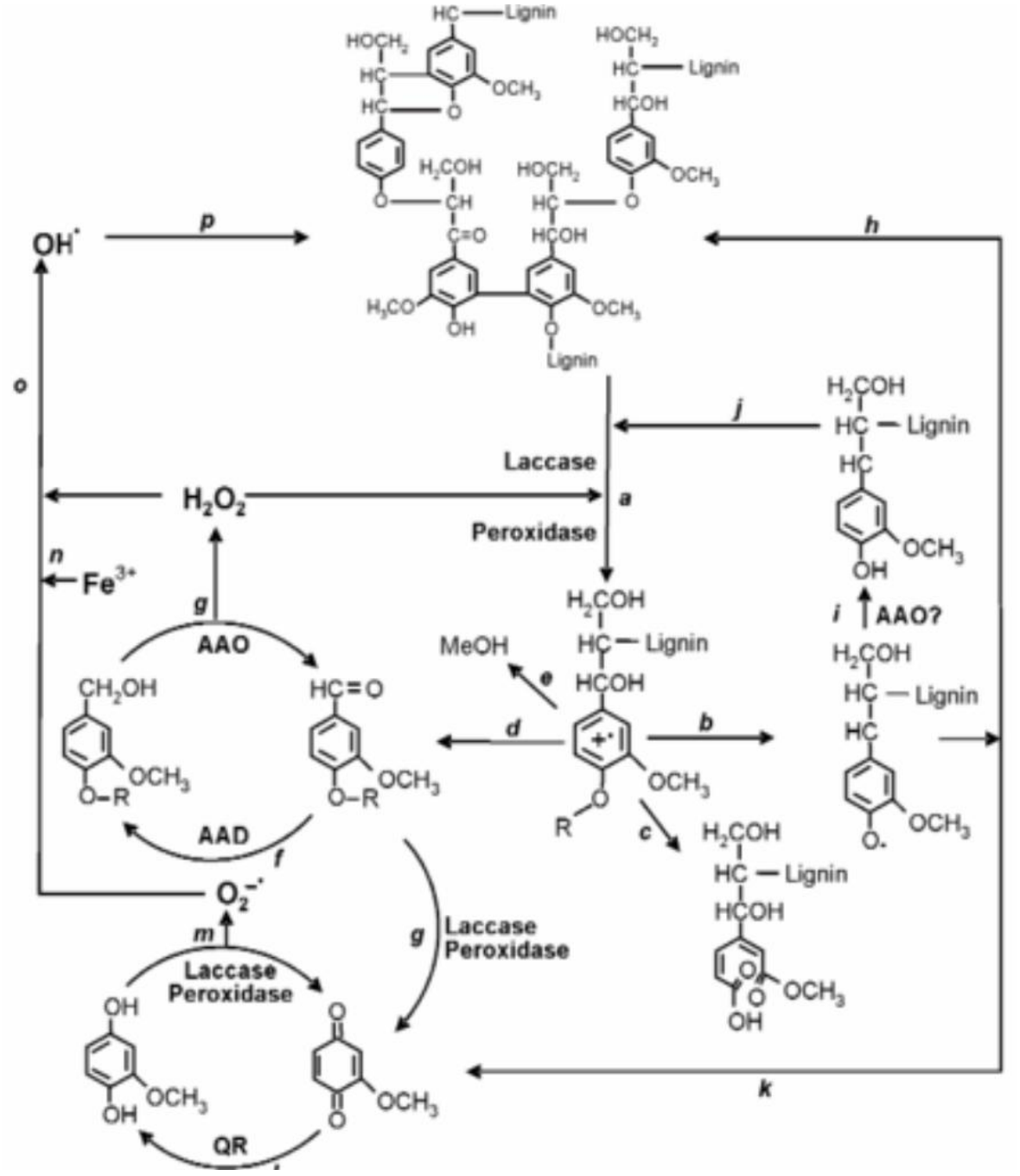


Fig. 17 Chemical and enzymatic degradation of lignin

Cellulase free xylanases active at high temperature and pH are gaining importance in pulp and paper technology as alternatives to the use of toxic chlorinated compounds (Viikari 1994, srinivas and Rele 1999). A treatment with xylanases can improve the chemical extraction of lignin from pulp (Bajpai *et al* 1994). This result in significant saving of chemicals required for bleaching thereby reducing the release of toxic chlorine compounds into the environment.

The xylanase treatment is normally carried out as an early step in the bleaching sequence, and it usually leads to the degradation of less than 10% of the xylan in a pulp. Xylanases cannot degrade or modify lignin directly. The mechanism for how xylanase bleaching works is not clear, but three main hypotheses are described below.

(1) Lignin covalently bond to Xylan (LCC) Lignin entrapped physically to xylan can easier be extracted from the fiber after xylanase treatment.

(2) Xylan that has re precipitated on the fiber surface and works as an obstacle for bleaching chemicals to enter fiber. Xylanase treatment partly removes the xylanase layer and open pores for bleaching chemicals to enter the fiber.

(3) Xylan contains hexenuronic acid that consumes bleaching chemicals. Xylanase treatment removes regions with high content of hexenuronic acid and there by consumption of bleaching chemicals is decreased.

On one hand, it has been observed that the kappa number, which reflects the lignin content of pulp, is decreased during the xylanase treatments. This suggests that xylanases contribute to delignification of the pulps. On the other hand, it has been suggested that xylanases might act by removing hexenuronic acid, which is a component of some xylan polymers, and which consumes bleaching chemicals.

Depending on the type of pulp, either one or both of these mechanisms may contribute to the bleaching effects observed. A third explanation is offered by the observation that some of the xylan dissolved during Kraft pulping of birch-wood under classical conditions reprecipitates on the fiber surfaces. These precipitates may trap some residual lignin, the removal of which could explain the bleaching effect of the xylanases.

SIGNIFICANCE OF BIOPULPING

However, the concept of biopulping is based on the ability of white rot fungi to colonize and degrade selectively lignin in wood and leaving cellulose fibers intact (Bajpai 2012).

Advantages of Biopulping

The most prominent benefit of fungal pretreatment is improved effects on cooking, leading to reduced kappa numbers/reduced active alkali charge and/or reduced cooking time after only 1–2 weeks of fungal treatment. Fungal pretreatment also reduces the pitch content in the wood chips and improves the pulp quality in terms of brightness, strength, and bleach ability. Hydrolysis of hemicelluloses must also be considered when lignocellulosic residues are subjected to biomass conversion. However, this will be determined by the pretreatment methods. Specifically in an alkali pre treatment method, a part of lignin will be removed and thus hemicellulose has to be degraded by the use of Hemicellulases (Hahn-Hagerdal *et al* 2006).which leads to separation of cellulose fibers.Cellulase-free xylanase preparations should be developed in industrial applications such as the prebleaching of kraft pulps in case of pulp and paper industry.

Disadvantages of Biopulping

The two main disadvantages of biopulping i.e., pulp darkening and 2 weeks incubation time, have limited its commercial deployment, but several effects of biopulping can be mimicked in the absence of organisms (Singh *et al* 2010).

A wide variety of environmental pollutants such as chlorophenolic compounds, polycyclic aromatic hydrocarbons (PAHs), cyanide as well as desulphurizing coal degraded by white rot fungi (Sedarati *et al* 2003, Baborová *et al* 2006, Çabuk *et al* 2006, Aytar *et al* 2008). Chlorinated phenols, recalcitrant compounds, are discharged from many chemical industries (Rubilar *et al* 2008) especially pentachlorophenol, 2, 6-dichlorophenol, 2,4,6 trichlorophenol, lindane, aldrin, 2,4-dichlorophenol, dichlorodiphenyltrichloroethane (DDT) have a widespread environmental concern because of toxicity and persistence (Dercova *et al* 2003, Furukawa 2003, Sedarati *et al* 2003, Kargi and Eker 2004).

Due to their powerful degrading capabilities towards various recalcitrant chemicals, white-rot fungi and their lignin degrading enzymes have long been studied for biotechnical applications such as biobleaching (Takano *et al* 2001), biodecolorization (Dias *et al* 2003) and bioremediation (Beltz *et al* 2001, Cheong *et al* 2006). Different fungi like *Phanerochaete chrysosporium, Candida maltosa, Pleurotus ostreatus, Lentinus edodes, Trametes versicolor, Pycnoporus cinnabarinus, Pleurotus sajor-caju* have been studied for biological treatment of chlorophenolics (Cameron *et al* 2000, Schultz *et al* 2001, Rodriguez *et al* 2004, Ünal and Kolankaya 2004).

After the first report by Viikari *et al* 2001 use of enzymes in paper industry has been revolutionized. A number of reports have been published on the application of hydrolyzing enzyme for biobleaching (Ahlawat *et al* 2007, Dhiman *et al* 2009). The majority of these investigations however, focused on the degradation of wood from a single tree species by

pure cultures of white-rot fungi. Luna *et al* 2004 and very few studied the effect of co-cultures on wood degradation.

Coculture/mixed culture and its significance in biopulping

Coculture/Mixed culture is the technique in which two or more organisms were allowed to grow together in the same medium. To obtain fungal coculture same size mycelial discs were removed from each of the fungal and placed at equal distance near to the margin of petridish containing media and incubated at desired temperature and incubation period then their growth and interactions were studied. These organisms may be benefitted or harmful or neutral with each other. Mixed fungal cultures could lead to a higher enzyme production through synergistic interactions but the final result seems to depend on the particular species combination or on the mode of interactions between species, and on the micro environmental or nutritional conditions in the substrate under colonization (Gutierrez-Correa and Tengerdy 1997).

Dual cultures are known to degrade wood more than monocultures (Watanabe *et al* 2003). Dual culture or co-culturing of fungi means oxidative stress to both fungal partners and acceleration of fungal metabolic switch to secondary metabolism stimulating wood decay and production of lignin degrading enzymes.

In natural environment it is possible for many fungi to live and grow nearing close proximity to each other. According to Rayner and Boddy 1988 the relationship between these fungi can be classified into competitive, neutralistic, and mutualistic interactions. The competitive interaction may be detrimental to either one or both of the species involved.

In the case of neutralistic interactions one or both partners neither benefited nor harmed and in mutualistic interaction either one or both the partners are benifited. Such benefits may result for different reasons like one organism may provide waste products or exudates as a resource for the other; the vegetative or reproductive development of one

organism may be stimulated by products from the other; or a complementary enzyme action may be achieved for both organisms.

Wood rotting fungi may show antagonistic interactions resulting in faster nutrition exploitation, or in parasitism, or may form deadlock interactions, where no hyphae of one species can enter the territory occupied by the other. Interactions may be synergistic, i.e. species can act in coordination to degrade the same substrate (Boddy 2000).

Antagonism a partial or complete suppression of activity or effect of other isolate by particular one is an important aspect to be considered for the co culturing experiments. Antagonist means one suppresses the effect of another. Antagonistic effect of fungi is the consequence of one fungus counteracting the effects of another fungus. Antagonistic property of fungi can be as the control of wood attacking fungi with fungal biocontrol agent.

Most widely studied genera of fungal biocontrol agent is *Trichoderma*, which has controlled several soil-borne pathogens under experimental conditions (Cook and Baker 1983). *Trichoderma* species are used in a wide range of commercial applications including the biological control of plant diseases (Howell 2003 and Harman 2006). On a malt agar medium, an isolate of *Trichoderma virens* completely inhibited growth of several white and brown rot fungi (Highley *et al* 1988).

Strains of the species *T. harzianum* has shown effectiveness when used in disease control caused by several fungi, including *Sclerotium rolfsii*, a widely distributed and highly destructive plant pathogen (Benhamou 1996), and *Sclerotinia sclerotiorum* affecting runner beans (Inbar 1996).

T. viride and *Gliocladium virens* have been effective in the control of *Phytophthora* ssp. causing cotton root disease. *G. virens* and *Trichoderma* sp. have been used to control *Fusarium oxysporum* and *Fusarium solani* and *Rhizoctonia solani* (Zhang *et al* 1996). *T. harzianum* produces antibiotics active against *Botrytis cinerea* (Bélager *et al* 1995). *T. viride*,

T. harzianum and *Trichoderma* sp. were able to stunt *Sclerotium cepivorum* growth the causative agent of white onion root rot (Kay and Stewart 1994). It has also been demonstrated that strains of *T. harzianum* and *G. roseum* are effective antagonists of *Pythium ultimum* and are used in pea rot protection (Steinmetz *et al* 1994).

Co-cultivation was a potential strategy in lingnocellulolytic biodegradation with producing high activity enzymes due to their synergistic action. (Qi *et al* 2011). Consortia of microbes are known to degrade lignocellulosic material in nature (Watanabe *et al* 2003). Fungal coculture aids to improve hydrolysis of lignocellulosic residues, and causes enhancement of product utilization which minimizes the need for additional enzymes in the bioconversion process (Dashtban *et al* 2009).

Mixed fungal cultures have many advantages compared to their monocultures, including improving productivity, adaptability and substrate utilization. Dashtban *et al* 2009). Two biopotential fungi which are not antagonist but compatible/synergistic of which one or both are selective in lignin degradation and producing xylanase enzyme would help in increasing the efficiency of biopulping by degrading lignin in an eco friendly manner and also would help in brightening the cellulosic fibers preventing yellowing of paper. The fungi which produce cellulase free xylanase enzyme is mainly used for the pulp and paper industry.

Synergistic interaction is a phenomenon in which interaction of two or more organisms resulting in overall effect which is greater than the sum of individual effect of any of them. Simultaneous action of xylanase and lignin degrading enzymes may prove to be a promising strategy to achieve higher degree of pulp bleaching. The action of xylanase would expose lignin which will be degraded and removed due to presence of lignin degrading enzymes, therefore leading in to improved level of delignification.

The recent interest in xylan-degrading enzymes comes from their potential use in the pulp and paper industry for bio bleaching has increased significantly in past few years(Atik *et*

al 2006) thus reducing the amount of toxic bleaching chemicals in plant effluents (Bajpai *et al* 1994, Ragauskas *et al* 1994). Fungal xylanolytic enzymes have received a great attention in last few years. Xylanases are produced by many different types of fungi such as *Trichoderma, Aspergillus*, *Penicillium, Schizophyllum, Aureobasidium* and *Talaromyces* spp. it was known that *Trichoderma* spp. are effective producers of xylanase (Gomes *et al* 1993, Kadowaki and Souza 1997) and number of *Aspergillus* spp have been reported to produce xylanase enzymes (Angayarknni *et al* 2006, Rizzatii *et al* 2004).

A good biopulping effect is obtained after incubation of wood chips with a selectively lignin degrading fungus for two weeks (Akhtar *et al* 1998). Conversion of both lignin and hemicellulosic hydrolytic products in a single process can be achieved by co-culturing two or more compatible microorganisms with the ability to utilize the materials. In fact, in nature, lignocellulosic residues are degraded by multiple co-existing lignocellulolytic microorganisms. The main drawback of co-culturing however is the complexity of growing multiple microorganisms in the same culture (Lynd *et al* 2002).

Interaction between different fungi as well as between fungi and prokaryotes can have an inducing effect on laccase activity (Ferreria Gregorio *et al* 2006, Chi *et al* 2007, Kleeman 2007). Several white rot and litter degrading basidiomycetes showed higher laccase activity in cultivation with another basidiomycetes species on plate and liquid medium (Iakovlers and Stenlid 2000, Baldrian and Snajdr 2006) even up to 25 fold. Stimulating laccase production between ligninolytic white rot fungi would be very useful in biopulping of wood as rightly indicated by Wang *et al* 2006.

The main aim of the present study was to identify two potential fungi which are not antagonist but compatible so as to suggest them for dual culture to increase the efficiency of biopulping. For natural enzyme production, fungal strains should be selected which are most efficient in the secretion of enzyme with required characteristics. The fungal combination has

to be such that one is ligninolytic and the other keeps up the quality of the cellulose fiber, especially the brightening property of the paper. Plant waste materials like Mandarin peels, groundnut shells, organic nitrogen sources and other nitrogen sources can very much raise the enzyme yields of various white rot fungi (Kachlishvili *et al* 2006, Vikineswary *et al* 2006). Also optimization in growth of the fungi and enzyme production is nowadays addressed by experimental design technologies considering variable parameters like nutrient concentration, inducers, agitation, pH, temperature and inoculums.

With an objective to obtain a better insight in to the potentiality of mixed cultures to increase production of ligninolytic and xylanolytic enzyme production the following main objectives have been laid down for the present study.

- Screening and identifying ligninolytic and cellulolytic and xylanolytic fungal isolates
- Check antagonistic activity of fungal isolates
- Assessment of cellulase free xylanase and lignin degrading enzyme activity
- Check the effects of incubation time, temperature and pH on enzyme activities
- Study the pattern of fungal degradation in *Eucalyptus* wood blocks
- Analyse Biochemical alterations/changes of wood components in the pretreated wood blocks
- Study on the enhancement of enzyme activity using different chemical enhancers and lignocellulosic substrates
- Evaluate the properties of fibers in pretreated wood blocks
- Suggest the optimized conditions for the biopulping using the best mixed fungal isolates