CHAPTER 1

INTRODUCTION

Many years ago, pulp and paper production has risen globally and still be continuously increasing because the demand for the use of paper products have greatly arisen and cannot be predicted. The number of wood pulp to be produced worldwide for the year 2010 is approximately 260 million tons. The increase of productivity of wood pulp to supply consumer demand directly affects on an increase of wastewater from the processes which highly potential impact on environment.

The process of wood pulp involves not only a large consumption of energy and chemicals, but also a conduct with high temperature and pressure, which are the main cause of such problems. Pollution of the effluent from pulp and paper manufactory has been focused particularly on the quality of wastewater from pulp bleaching process. The effluent contains not only chlorinated and phenolic compounds but also have high darken color due to lignin and derivative substances such as ligninosulfonate which was removed from kraft pulp. Most of organic substances contained in the effluent, such as sulphur compound and sodium hydroxide looks potentially have high toxicity and harmful to environment and public health. Therefore, many authorities which involve in environment protection have closely concentrated in this problem and provided the environmental regulation to force the paper mills having more regard for elimination of toxic substances in the effluent before discharged to environment. Although there is the environmental regulation to solve this problem, the pulp and paper factories still release unpleasant smell of sulphur compounds into the air and discharge wastewater which enhance eutrophication and have toxic effects on the surrounding biota. Moreover, numerous chlorinated organic compounds from during the chemical pulp bleaching have been particularly concentrated continuously.

The different strategies can be applied to solve the toxicity problem in wastewater including : (i) utilizing microorganisms and their enzymes, such as peroxidase and laccase instead of numerous chemicals in pulping and bleaching processes and (ii) remove or at least reduction of organic substances (detoxicity) and dark color (decoloration) in the effluent by biotechnological methods. Among wide variety of microorganism which implicated in lignin degradation, basidiomycete fungi causing white rot of wood have been intensively studied as being typical lignin degrader (Higuchi, 1993). Therefore the cooperation between application of white rot fungi and the basic knowledge involves in biotechnology have been expected to be a promising alternative method to solve or improve environmental problems which caused by pulp and paper industry.

LITERATURE REVIEW

Pulp and paper industry and impacts on environment

Traditional pulp and paper production, conventional pulp bleaching use a variety of chlorine derivatives as bleaching agents. Almost half of lignin in the kraft pulp are bleached by chlorine and oxides, and extracted with alkali. The advantages of using chlorine are simple, cheaper, selective, and effectively to remove lignin. Bleaching with chlorinated chemicals usually starts with an acid treatment with elemental chlorine at low temperature, and pH. During chlorination, wood components, lignin and carbohydrates are structurally modified and degraded to obtain chlorinated derivatives. This stage is followed by an alkali extraction at high temperature, and pH. In the extraction stage, chlorinated and oxidized lignin are not soluble in the acidic chlorination stage, but are solubilized and dissolved into the spent liquor. The final bleaching is achieved by using oxidizing chemicals, usually chlorine dioxide and hydrogen peroxide. The substances dissolved in the latter stages are more strongly oxidized, and a pollution comes from these stages are minor. The pulp mill in Scandinavia and North America have stopped using chlorine bleaching so that they meet government regulation on adsorptive organic halide (AOX) discharge. In some countries such as India, bleaching is still being done with chlorine. Chlorine dioxide is used by very few mills for viscosity protection in the first bleaching stage (10 to 15% substitution) and for the brightening in the final bleaching stages. Oxidative extraction and peroxide bleaching are also being tried in some mills (Nagarathnamma and Bajpai, 1999).

Traditional lignin removal agents which have been extensively used in pulp and paper industry up to date are not only chlorine and its compounds, but also other chemicals such as sodium hydroxide, sodium sulfate, and hydrogen peroxide. Type and amount of uses are varied and dependent on pulp and mill type and regulatory limits which are provided by laws in each countries. Table 1.1 is an example for type and amount of chemicals uses in pulp bleaching stage from pulp and paper mill in Thailand.

Table 1.1 Amount of chemicals uses of pulp bleaching per ton of pulp

Type of chemical	Dosage (lb)
Chlorine	125
Sodium hydroxide	50
Chlorine dioxide	16
Sodium sulfate	14

Based on data from Sarangbin (1980)

The toxicity of the above chemicals looks full of danger for the environment and people who live around pulp mills. Chlorine or liquid chlorine are strongly unpleasant smell and dangerous oxidizing agents when combined with acetaldehyde metal ion which lead to be strongly oxidizing reaction. When it reacts with alcohol, ether, hydrocarbon, acethylene, and ammonia, chlorine will release unpleasant vapour to the lung and eye which leads to be sore eye. Sodium sulfate is directly toxic to the skin and the respiration system. Sodium hydroxide impacts severe effects on environment and biota because of the severe alkalinity.

Most of mills are not willing to recycle bleached plant effluent to the chemical recovery system due to the corrosive nature of chloride ion and the substantial dilution of the chemicals to be recycled. Chlorinated organic compound is produced during pulp bleaching not only use an oxygen demand (biochemical oxygen demand [BOD] and chemical oxygen demand [COD]), but also cause effluent color and toxicity (Kringstad, 1984). Chlorinated organic subsatnces in spent bleaching liquor is also responsible for mutagenicity of the effluent (Nagarathnamma, 1999). The low molecular weight fractions of the chlorolignins are the main donator to the effluent BOD and acute toxicity. The high molecular weigh chlorinated compounds obtained small amounts of BOD and acute toxicity due to their inability to pass through cell membranes. They are the major donators to effluent color, COD, and chronic toxicity (Eriksson *et al.*, 1985). Color is not only aesthetically unacceptable, but also inhibits the natural process of photosynthesis in stream

(Kringtad and Linstrom, 1984). These lead to disrupt the chains reaction of adverse effects on the aquatic ecosystem because the growth of primary consumers as well as secondary and tertiary consumers is adversely affected. Discharge of untreated or partially treated wastewater from pulp and paper mills results in persistence of color in the receiving body over a long distance. Under natural conditions, these compounds are slowly degraded to various chlorinated phenolics, which may be methylated under aerobic conditions. The low molecular weight phenolics and their methylated counterparts (which are lipophilic) cause toxicity and are bioaccumulable in fish (Eriksson *et al.*, 1985). However, Archibald *et al.* (1993) reported that the toxic levels of low molecular weigh chlorinated compounds are not accumulated during the natural degradation of chlorolignins but really appear to stimulate the growth of algae and primary consumers, probably because of the nutrient composition.

About 75% of the dissolved organic material, 60% of the COD loading, 40-50% of the organically bound chlorines, and 80% of the color-imparting substances were found in the bleach plant effluents. Therefore, a treatment method that can degrade, dechlorinate, and decolorize extraction-stage effluent can deal with most of environmental pollution problems associated with bleach plant effluents (Nagarathnamma and Bajpai, 1999).

In kraft pulp bleaching 90-95% of lignin was removed and the remaining of lignin 5-10% in the bleached pulps is responsible for the blown color of kraft pulp and paper (Eriksson and Kirk, 1985). The effluent produced during chlorine bleaching is the major contributor to high toxic components in wastewater from the factories. It contains highly modified lignin and chlorolignins of different molecular weight together with phenol and chlorinated phenol (Kringstad and Lindstrom, 1984 ; Eriksson *et al.*, 1985), which are toxic to the environment.

Most of brown color in chlorine bleached effluents is due to quinones. On the other hand, the effluent derives from the first alkali extraction stage containing conjugated lignin structure. Therefore, the decoloration and removal of these high molecular weight compounds are necessary. Bacteria cannot be able to degrade these compound but fungi, especially, the white rot group due to the ability to degrade these chlorolignin (Eriksson *et al.*,1990).

Effluent from the chemithermomechanical pulping (CTMP), shown in Table 1.2, is a further refinement of thermochemical pulping (TMP) which involves pretreatment of wood chips with sodium sulfite (about 2% on dry wood) at pH 9-10 or sodium hydroxide (with hydrogen peroxide in alkaline peroxide method). Then, it was steamed at 130-170°C, and finally refined (Bajpai *et al.*, 1999). Table 1.3 shows the characteristics of CEDD bleaching sequence (where C is chlorination, E is alkaline extraction, and D is chlorine dioxide) from a pulp mill in India.

Table 1.2 Characteristics of bleached Chithermomechanical pulping (CTMP) wastewater

Parameter	Value
pH	6.5
COD total (mg/l)	9300
COD soluble (mg/l)	5030
TSS (g/kg)	2.45
VSS (g/kg)	1.98

Based on data from Nagarathnamma and Bajpai (1999)

As the previous serious problems, the environmental protection of effluents from pulp mill have been more concrened. The environmental regulation has been provided to force the mills in Europe, Canada and Japan, and recently in the USA, to eliminate the toxicity through the combination of process modification and effluent treatment technology before discharge to environment (Bajpai *et al.*, 1999). The regulatory emission limits for pulp and paper mills in each countries are shown in Table 1.4 to 1.11

From the regulatory environmental protection, biotechnology is a potentially promising means for reduction of environmental problems. Many developed countries and environmental realizable countries have particularly focused on the applications of white rot fungi. The researches involve pollutant environmental protection have been increasingly studied each year. Some of them were summarized in the next section

Table 1.3 Characteristics of bleach plant extraction-stage effluent in India

Parameter	Value for alkali Extraction-stage effluent
Color (PCU)	7266 ± 251.6
COD (mg/l)	1359 ± 52.6
BOD (mg/l)	418 ± 20.2
AOX (mg/l)	58.6 ± 1.52
EOX (mg/l)	1.33 ± 0.11
pH	10.2 ± 0.25
Alkalinity (mg/l as $CaCO_3$)	726 ± 64.2
Total solids (mg/l)	3340 ± 50
Dissolved solids (mg/l)	3000 ± 00
Suspended solids (mg/l)	253 ± 50.3

Based on data from Nagarathnamma and Bajpai (1999)

Table 1.4 Regulatory emission limits for pulp mills in Sweden

Parameter	Discharge limit (kg/ton of pulp)		
	1991	1995	1999
BOD	10-20		
COD	10-20		
TSS	40-70		
TOCI Softwood	2.0	1.0	0.5
Hardwood	1.0	0.5	0.3
AOX Softwood	2.5		
Hardwood	1.3		
Nitrogen	0.3		
Phosphorus	0.1		

Based on data Bajpai *et al.* (1999)

Table 1.5 Regulatory emission limits for pulp and paper mills in Germany

Mill type	Discharge limit (kg/ton of pulp)		
	BOD	COD	AOX
Paper mills			
Wood-free unsized	1	3	0.04
Wood-free sized	2	6	0.04
Wood-free highly refined	3	9	0.04
Recycle fiber-based	-	5	0.012
Chemical pulp mills	5	70	1.0

Based on data from Bajpai *et al.* (1999)

Table 1.6 Discharge limits for pulp and integrated paper mills in Portugal

Mill type	Discharge limit (kg/ton of pulp)			
	TSS	BOD	COD	AOX
Unbleached kraft pulp				
1991	2.5	5	-	-
1996	1.5	3	35	-
Bleached kraft pulp				
1991	10	9	-	-
1996	3	6	50	1.5
Bleached sulfite pulp				
1991	12.5	45	-	-
1996	6	25	120	1.5
Integrated kraft liner				
1991	4	6	-	-
1996	2	4	30	-

Based on data from Bajpai *et al.* (1999)

Table 1.7 AOX discharge limits in Canada

Province	Year	AOX limit (kg/ton of pulp)
Ontario	1991	2.5
British Columbia	1992-1993	2.5 (Softwood) 3.8 (Hardwood)
Quebec	1992	2.5 (Softwood) 1.5 (Hardwood)
	1995	1.5 (Softwood) 0.5 (Hardwood)

Based on data from Bajpai *et al.* (1999)

Table 1.8 Discharge limits for pulp and paper mills in Japan

Parameter	Value	
	Maximum	Daily average
BOD (mg/l)	160	120
COD (mg/l)	160	120
TSS (mg/l)	200	150
AOX (mg/l)	1.5	

Based on data from Bajpai *et al.* (1999)

Table 1.9 Effluent standards for pulp and paper industry in India

Mills type	Discharge limits			
	BOD (mg/l)	COD (mg/l)	TSS (mg/l)	TOCI (kg/ton prod)
Large pulp and paper	30	350	50-100	2
Newprint/rayon grade	30	350	50-100	2
Small pulp and paper				
Agrobased	30	-	100	-
Waste-paper based	30	-	100	-

Based on data from Bajpai *et al.* (1999)

Table 1.10 Effluent limitation of pulp mill in China

Parameter (kg/ton)	First-class receiver	Second-class receiver	Third-class receiver
BOD	7.2	28.8	120
COD	16.8	48.0	96
TSS	24.0	84.0	192
AOX	1.5	2.5	-

Based on data from Bajpai *et al.* (1999)

Table 1.11 Effluent discharge standard in South-East Asia

a) Thailand

Parameter (mg/l)	Pulp mill	Paper mill
BOD	20	20
COD	120-500	120
TSS	50-150	50-150
TDS	3000-5000	3000-5000
TKN	100-200	100-200

b) Indonesia

BOD (mg/l)	50-95	35-50
(kg/ton)	50-100	60-90
COD (mg/l)	3-9.5	2-6.5
(kg/ton)	120-350	100-175
TSS (mg/l)	7.2-29.75	5.6-10
(kg/ton)	75-100	80-100

c) Malaysia

Parameter (mg/l)	Standard A	Standard B
BOD	20	50
COD	50	100
TSS	50	100

d) Philippines

Parameter	Protected water catagoty II	Inland waters class C
BOD (mg/l)	30	50
COD	case by case	case by case
TSS (mg/ml)	50	70
TDS (mg/l)	1000	-
Color (PCU)	100	150

Based on data from Bajpai *et al.* (1999)

Lignin

Lignin is a phenylpropanoid structural polymer of vascular plant which gives the plants rigidity and binds the fiber and cell walls together. Lignin also decreases water permeation across cell wall of xylem tissues and provides the wood more resistant to attack by microorganism (Kirk, 1971; Vance *et al.*,1980). Although quite resistant to microbial attack, lignin is ultimately degraded to humus water, and carbon dioxide following the death of plant tissue (Hurst and Burges, 1987; Zeikus, 1981). The humus found in the upper layer of soils is important for the structure of the soil and increase aeration and moisture-holding capacity. The humus also functions as a basic ion exchanger and is able to store and release nutrients to the surroundings, that is, to growing plants and trees (Chistman and Oglesby, 1971).

Next to cellulose, lignin is the most important renewable material. However, it also constituted a barrier that must be bleached before most cellulose is accessible to enzymes. Lignin is a hindrance for efficiently utilizing cellulose in the production of ethanol, sugar, and protein (Eriksson, 1985; Eriksson and Kirk, 1985). For the biotechnical production of the useful products from lignin and for the improvement of existing pulping and bleaching processes in the pulp and paper industry, a detail knowledge of the basic mechanisms of biological lignin degradation is necessary. Similarly, in the upgrading and delignification of straw and sugarcane bagasse for cattle feed or delignification of wood for paper production, a through knowledge of ligninolytic organisms is needed.

In the late 1960, the chemical structure of lignin became clear and the complexity of this structure was analyzed and found that lignin is distributed throughout the secondary wall and compound middle lamella, but the greatest concentration is in the middle lamella approximately 35 $^{\circ}$A thick (Westermark, 1982). The secondary wall constitutes a large proportion of the total cell wall area, and most (60-80%) lignin is located in this region (Fergus, 1969, Saka and Thomas 1982a,b). The distribution of wood components and lignin within the cell wall was shown in Fig. 1.1. According to Higuchi (1985) report that "lignin precursors are synthesized in Golgi bodies and/or endoplasmic reticulum, transported to the wall via vesicles, and dehydrogenatively polymerized to lignin by peroxidase bound to cell walls".

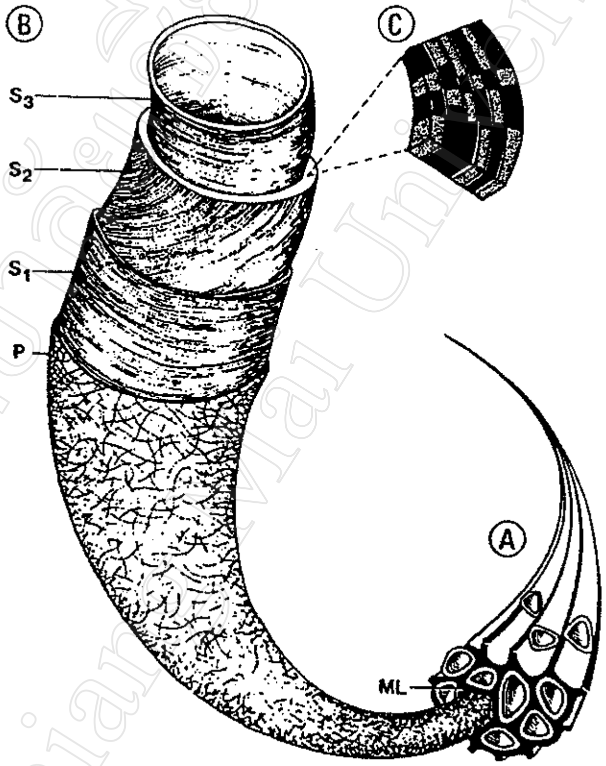
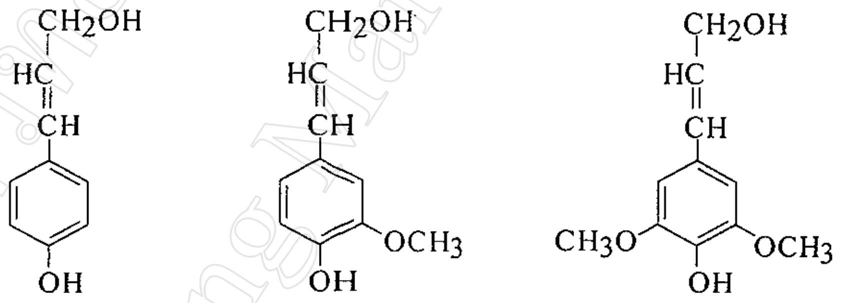


Fig. 1.1 The distribution of wood components and lignin within cell wall. A; tracheids, B: cell wall layers and C; ultrastructural arragement of lignin and carbohydrates in the secondary wall. ML; Middle lamellar, P; primary wall, S1, S2, S3; layer of secondary wall. The lignin-hemicellulose matrix (black), hemicellulose (white), and cellulose fibrils (stippled) (Eriksson *et al.*, 1990).

Lignin, which is a three dimentional polymer of one or more phenyl propanoid monomers, such as coumaryl, coniferyl, and sinapyl alcohol (Fig.1.2). The basic structural unit of lignin may be substituted in two or three positions: the addition of one methoxyl group to phenol ring results in a guaiacyl unit and the addition of two methoxyl groups results in syringyl unit. Hard wood lignin contains varying ratio of syringyl and guaiacyl, whereas coniferyl consists of primarily guaicyl lignin (Fengel and Wegener, 1983) which was corresponding with Paterson's report (1984) that there are equal amount of coniferyl and sinapyl alcohol (46%) and a minor amount of *p*-coumaryl alcohol (8%). On the other hand, a typical softwood (gymnosperm) lignin contains mainly coniferyl alcohol, some *p*-coumaryl alcohol, but has no sinapyl alcohol (Sakakibara, 1980).



p-coumaryl alcohol Coniferyl alcohol Sinapyl alcohol

Fig 1.2 The lignin precursors *p*-coumaryl alcohol, couniferyl alcohol and sinnapyl alcohol (Eriksson *et al.*, 1990).

Three lignin precursors form a macromolecule of lignin was shown in fig. 1.3 which is schematic formula for a section of spurce lignin (Adler, 1977). There are three major intermonomer linkages in lignin: (1) the arylglycerol-β-aryl ether structure which connects about half of the phenylpropane unit in spurce lignin and about 60% of these units in birch lignin (Adler, 1977); (2) the phenylcoumaran structure, which comprises 9-12% of the phenylpropane units in spurce lignin and about 6% of those in birch lignin; and (3) biphenyl structure, which involves 9.5-11% of the units in spurce lignin and 4.5% of birch lignin. There are many linkages bound to be the strong structure of lignin, which was not so easy to break out.

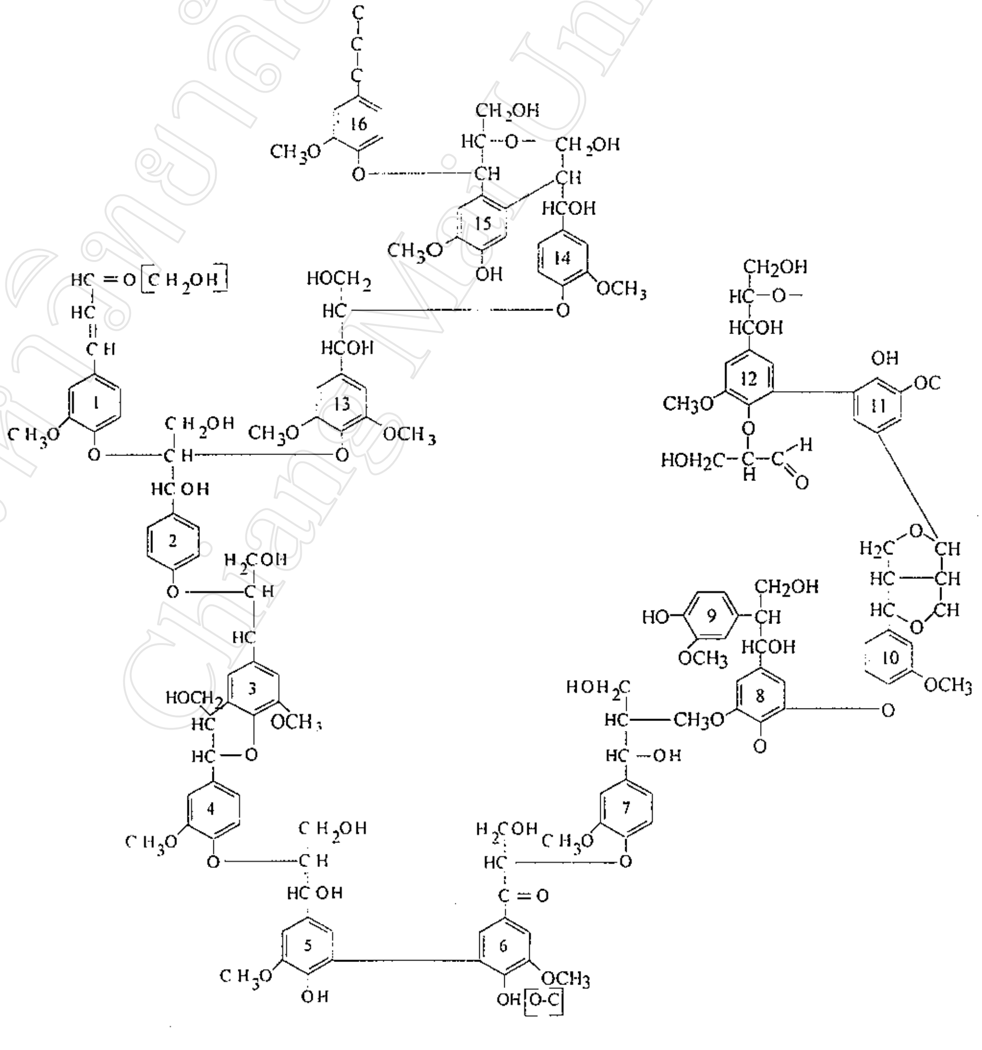


Fig 1.3 The structure of spurce lignin (Adler, 1977).

The nature of residual lignin in kraft pulp

Understanding the mechanism of biological delignification was studied in *T. versicolor* requires some understanding of lignin structure. Kraft pulping chemically degrades and dissolves the middle lamella and primary wall lignin that cements together the fiber in wood and most of the lignin embedded in the secondary wall. During normal kraft cooling, 90-95% of the lignin is removed from the fiber walls. The residual lignin has been extensively modified by cleavage of ether bond, side chain shortening, oxidation, and condensation reactions between lignin molecules and lignin or polysaccharides (Gierer, 1985; Hosoya, 1992). The syringyl lignin in hard wood is more sensitive to cleavage reactions and much less probable to condensation reactions than the guaiacyl nuclei. As a result of residual kraft lignins contain very few syringyl nuclei (Chiang and Funaoka, 1990). Cleavage of alkyl-aryl ether (i.e. α-O-4' and β-O-4', structure I in Fig. 1.4) is the major depolymerizing reaction during kraft pulping (Gierer, 1985). Carbon-carbon bonds (e.g. β-5' and 5-5', structure III and IV in Fig. 1.4) are relatively stable (Hosoya, 1992). The major condensation reactions between lignin units lead to diphenylmethane links (structure V and VI in Fig.1.4, Gierer, 1985). The residual lignin of hardwood kraft pulp contains 66% of dephenylmethane-linked guaiacyl units, 26% of otherwise condensed guaiacyl units, 6% of non-condensed syringyl units, and 2% of non-condensed guaiacyl units (Chiang and Funaoka, 1990b). About one-third of aromatic rings in kraft residual lignin have free phenolic groups (Hosoya, 1992). These peripheral rings can be studied by methylation-permanganate oxidation in a softwood kraft pulp, they consisted of 32% uncondensed guaiacyl rings, 15% rings condensed through the 5-position, 4% rings condensed through the 6-position, 26% biphenolic biphenyl (5-5') structures, and 16% biphenyl ethers 4-O-5', structure VII in Fig. 1.4 (Gellerstedt and Lindfors,1984). NMR analysis confirmed that the content of aromatic rings condensed through their 5-position increase in residual lignin during kraft pulping. Half of the aromatic rings were still involved in ether linkages at the end of pulping (Leary *et al.*, 1988).

Ether linkages between the α-position of lignin side-chains and the primary hydroxyl of a sugar molecule (structure II in Fig. 1.4) are markedly stable under kraft pulping conditions. These linkages lead the lignin unit contains none of free phenolic hydroxyl and the sugar contains no a free reducing end (Taneda *et al.*, 1987). Such linkages between lignin and carbohydrate are present in wood before pulping and during kraft pulping (Minor, 1986).

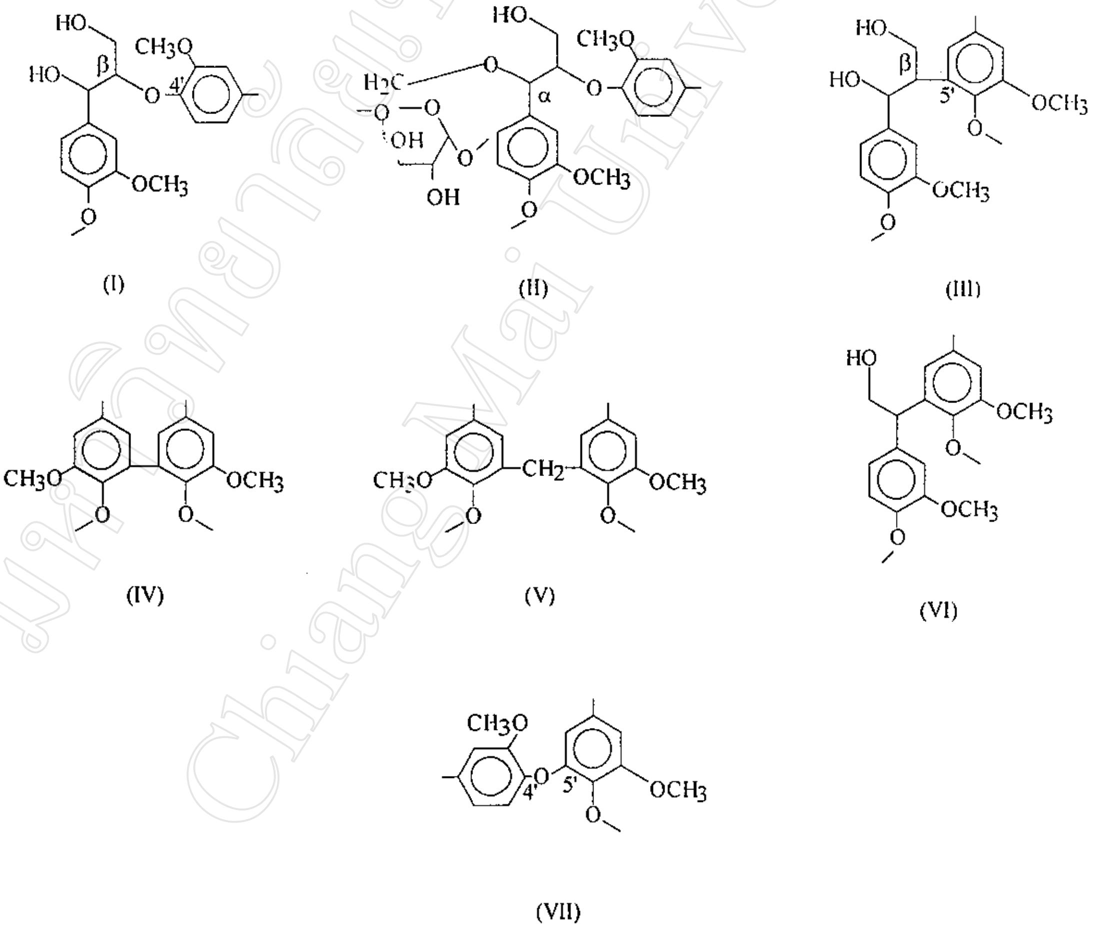


Fig. 1.4 Dominant substructures in the residual lignin of kraft pulps. I. *β*-O-4' alkyl-aryl ether. II. α-ether linkage to carbohydrate. III. *β*-5' carbon-carbon linkage. IV. 5,5'-biphenyl linkage. V. Diphenylmethane linkage. VI. Another type of diphenylmethane linkage 4-O-5' biphenyl ether (Paice *et al.*, 1993).

Both the condensation reactions within lignin, which increase the degree of polymerization and the linkages between lignin and carbohydrate, are thought to promote retention of the residual lignin within the pulp fiber walls. Since the residual lignin becomes soluble in alkali when the pulp polysaccharides are enzymatically digested (Jiang *et al.,* 1987; Paice *et al.*, 1993). Physical entrapment of the lignin by the polysaccharide chains or covalent attatchment to the insoluble polysacharides seem to be more important than intrinsic insolubility of the lignin.

Despite the chemicals removal of lignin during kraft pulping but the residual lignin is still sensitive to biodegradation by *T. versicolor* or other white rot fungi. One of the early events is the release of methanol from the methoxyl groups on rings bearing a free phenolic hydroxyl group (Fig. 1.5) (Paice *et al.*, 1993; Reid and Paice, 1994). Under the influence of the fungus, some of the residual lignin is solubilized and its smaller fraction is mineralized (Reid and Paice, 1994).

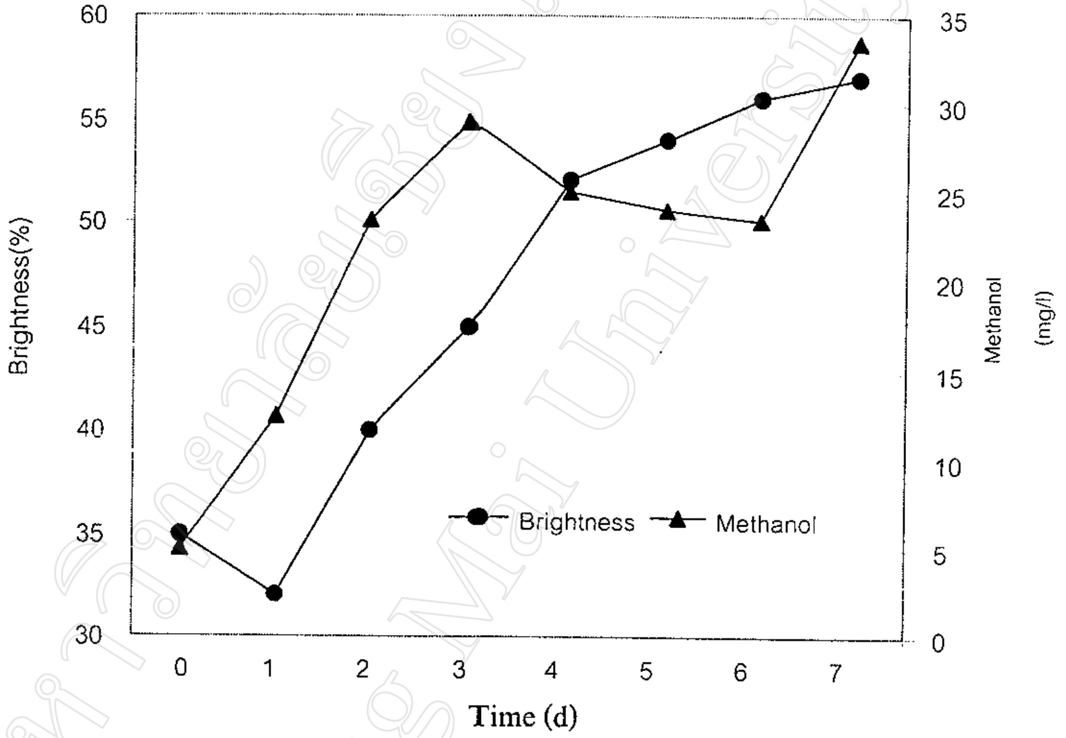


Fig. 1.5 Demethylation and bleaching of hard wood kraft pulp by manganese peroxidase (Paice *et al.*, 1993).

Relation of white rot fungi and basidiomycetes

Three types of wood decay have been distinguished including brown rots, white rot, and soft rot. Brown rots attack on the carbohydrate, cellulose and hemicellulose, but there is little effect on lignin. White rot fungi degrade wood either by simultanous attack on lignin and polysaccharides or by preferentially degrade lignin. The brown and white rots penetrate deep into the wood, but soft rots are more noticeable near the surface and have no effective on lignin.

Lignin is a natural polymer of cell wall that provides strength to wood. White rot fungi, which use cellulose as a carbon source, proceed the unique ability to degrade lignin completely to carbon dioxide to access the cellulose molecule. Although this ability has been acknowledged for many years, only recently has been begun to understand the mechanisms of lignin degradation by the whole enzymes from white rot fungi.

The lignin degradation enzyme system of white rot fungi is extracellular and unusually nonspecific. Peroxidase and laccase, which are secreted by the fungi, catalyze reaction of nonspecific free radicals, resulting in the depolymerization and degradation of lignin. Even though lignin is a naturally highly oxidized polymer, it can finally be completely oxidized to carbon dioxide by white rot fungi. The understanding of environmental pollutant degradation by white rot fungi is the main purpose to be achieved. The extracellular biodegradation system explains that fungi are quite resistant to the toxic or mutagenic chemicals. Scientists expect that an understanding of mechanism of wood degradation by white rot fungi will be applied to the environmental protection.

The largest group of white rot fungi belongs to the basidiomyctes, which are the best lignin degraders among all of the known microorganisms. Most of those species, which have dikaryotic hyphae, have clamp connections along the septate mycelium (Fig.1.6). These structures are unique characteristics in the basidiomycetes (Eriksson *et al.*, 1990).

Many different forms of clamp connection may be found. Fig. 1.7 shows the classification of basidiomycete in Fungi Kingdom.

Wood decayed by basidiomycetes has various color changes occurred after advanced stages of the decomposition. The complex structures of lignin are reduced to simple forms by white rot fungi. From these reasons, white rots decay wood become light or can be bleached white from dark brown color (Eriksson *et al.*, 1990). From all of the specific importance of basidiomycetes or white rot fungi on lignin leads to be increasingly focused on their enzymes to apply in the modification process and effluent treatment of pulp and paper manufactories.

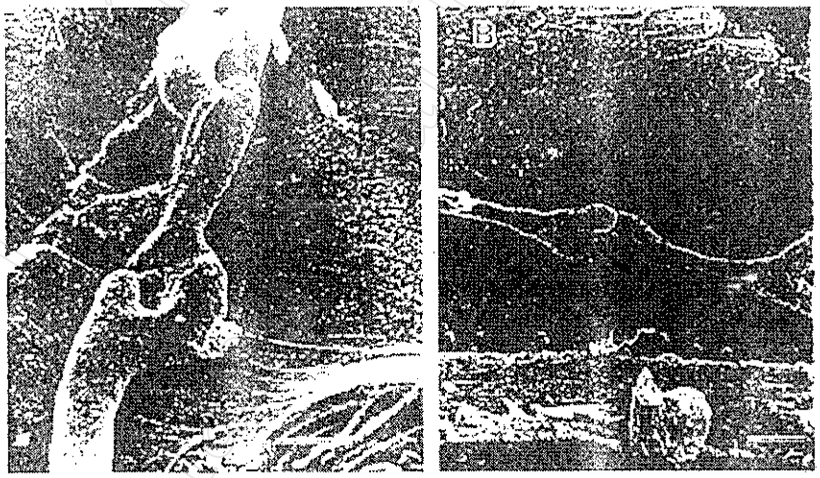


Fig. 1.6 Hypha of basidiomycete with clamp connection of *Coriolus versicolor*, a white rot fungus (A) and *Poria placenta*, a brown rot fungus (B) (Eriksson *et al.*, 1990).

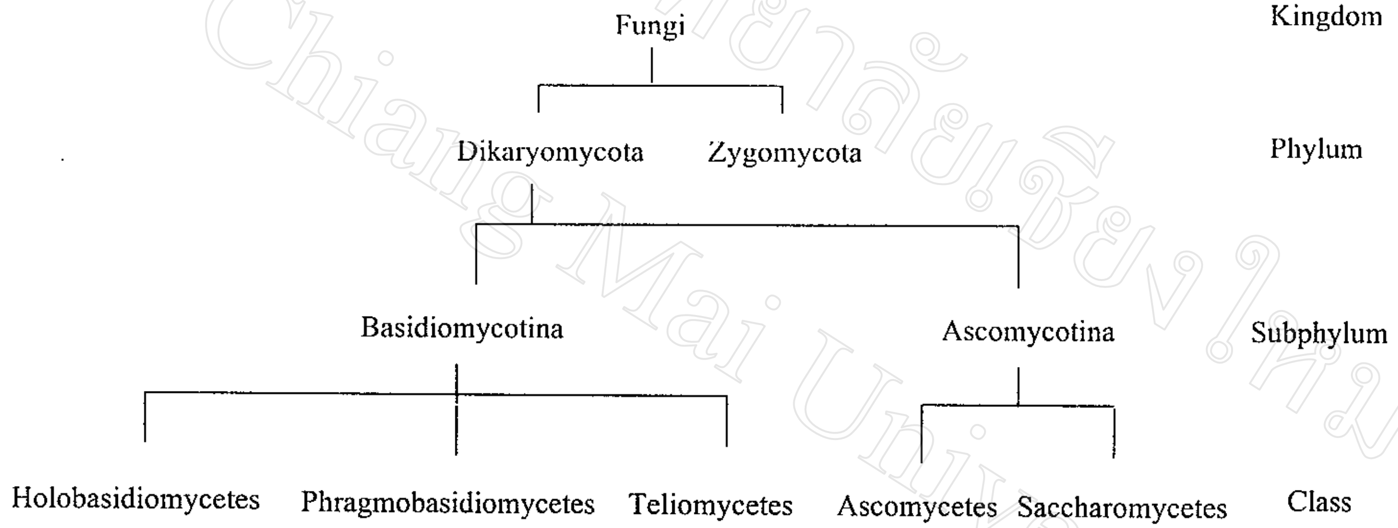


Fig. 1.7 Classification f basidiomycete fungi (Sa-ad-sud, 1998)

Roles of lignin degrading enzymes in kraft pulp bleaching

Since the applications of xylanase in pulp bleaching was found to be an essential step in demonstrating that enzymes are efficient technological means for pulp bleaching. Thus the enzymes from white rot fungi belong to lignin degrading enzymes have been particularly attracted and are being developed to increase pulp brightness, to reduce the chemical using and improve the quality of wastewater. The most important lignin degrading enzymes are lignin peroxidase, manganese peroxidase and laccase. Several reports suggested that these enzymes were useful in kraft pulp bleaching (Bourbonais *et al.*, 1992; Bourbonais *et al.*, 1995). All of ligninolytic enzymes are oxidative enzymes, which are produced by white rot fungi The ligninolytic enzymes consist of lignin peroxidase, manganese peroxidase and laccase.

Lignin peroxidases

Lignin peroxidase (LiP) or ligninase are hemeproteins, which are able to catalyze the oxidation of nonphenolic aromatic ring in lignin to cation radical in the presence of hydrogen peroxide. Fig. 1.8 shows the relationship of veratryl alcohol and lignin peroxidase, however the actual formation of this cation radical has not been clearly demonstrated yet (Harvey *et al.*, 1986).

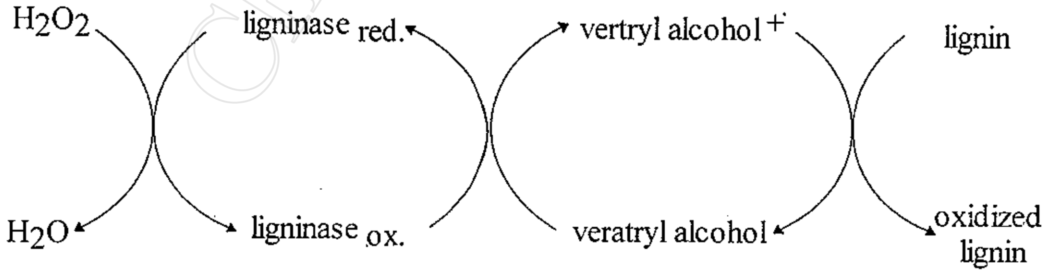


Fig. 1.8 Hypothetical scheme for lignin degradation by lignin peroxidase with veratryl alcohol as mediator (Harvey *et al.*, 1986).

Manganese peroxidase

Manganese peroxidase (MnP) was first discovered in 1984 in *Phanerocheate chrysosporium* (Kuwahara *et al.*, 1984; Gold and Glenn, 1988). The enzyme has been found in many other ligninolytic fungi (Tuor *et al.*, 1995) including *T. versicolor* (Johansson and Nyman, 1987; 1993). Manganese peroxidase oxidizes phenolic compounds in lignin. They require Mn^{2+}, which is oxidized to Mn^{3+} in the presence of chelators and hydrogen peroxide. Mn^{3+} is the real oxidizing agent that attacks lignin molecule (Fig. 1.9). During the bleaching of kraft pulp fiber by manganese peroxidase, oxalate and several chelators are biosynthesized. The biosynthesis requires manganese as a primary substrate.

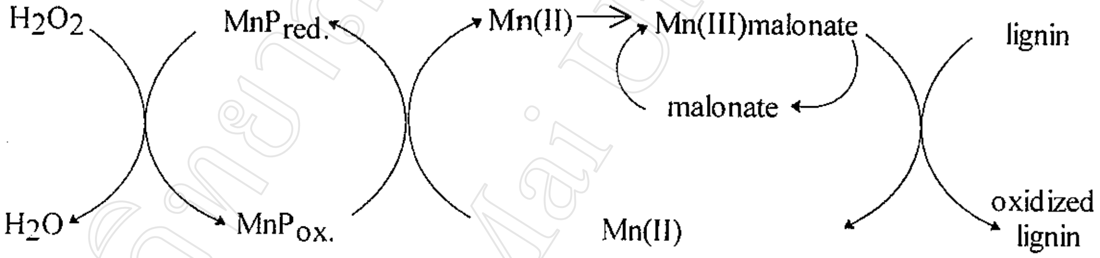


Fig.1.9 Oxidative pathway for catalytic action of manganese peroxidase on lignin (Harvey *et al.*, 1986).

Initially it was thought that the lignifying capability of MnP was limited to phenolic subunits of lignin (Kuwahara *et al.*, 1984). Lignin peroxidase was required for complete fungal depolymerization of native lignin, where nonphenolic linkage predominate. Subsequently, it was recognized that MnP oxidized nonphenolic compounds (Wariishi *et al.*, 1989), and that substantial MnP catalyzed depolymerization of dehydrogenation synthetic lignin is possible (Wariishi *et al.*, 1989). Recently, oxidation of veratryl alcohol thought to be a unique reaction of lignin peroxidase, has been reported with MnP *Lentinus edodes* (Buswell *et al.*, 1995). It may be that MnP from different organisms, or isozymes from the same organism, have different oxidizing capabilities.

Laccase

Laccase or phenoloxidase was first obtained from the juice of the Japanese lacquer tree (*Rhus venicifera*), which was a member of the important lignin degrading enzymes including lignin peroxidase and manganese dependent peroxidase. Laccase (benzenediol: oxygen oxidoreductsaes; E.C. 1.10.3.2) used oxygen molecule as a co-substrate. The enzyme oxidize phenolic subunits in lignin and simultanously reduces oxygen to water (Fig. 1.10). Its applications are frequently used in paper pulp bleaching to remove lignin from unbleached kraft pulp and reduction of toxicity in wastewater from factories.

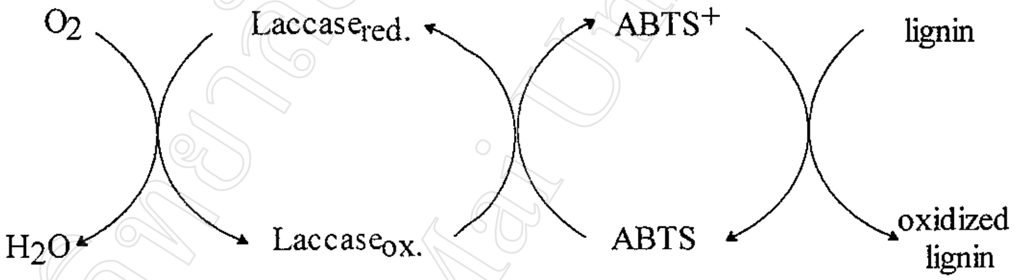


Fig. 1.10 Oxidative pathway for catalytic action of laccase on lignin (Harvey *et al.*, 1986).

The substrate range of laccase can be extended to nonphenolic compounds. Fig. 1.11 shows simple reactions catalyzed by laccase and nonenzymatic reaction. Laccase acts on lignin subunits or macrophenolic compound (Fig. 1.12). Laccase does not only catalyze the reduction of oxygen to water of *p*-diphenol to *p*-quinone, but can also cleave between the C_α- and the C_β-carbon in the side chain. C_α-C_β cleavage of compound I in Fig 1.12 was probably similarly catalyzed by horseradish peroxidase (Katayama and Higuchi, 1989). Laccase is also defined to be polyphenol oxidase (PPO), but its unique ability to catalyze *p*-diphenol to *p*-quinone can be used to distinguish laccase activity from the other members of PPO.

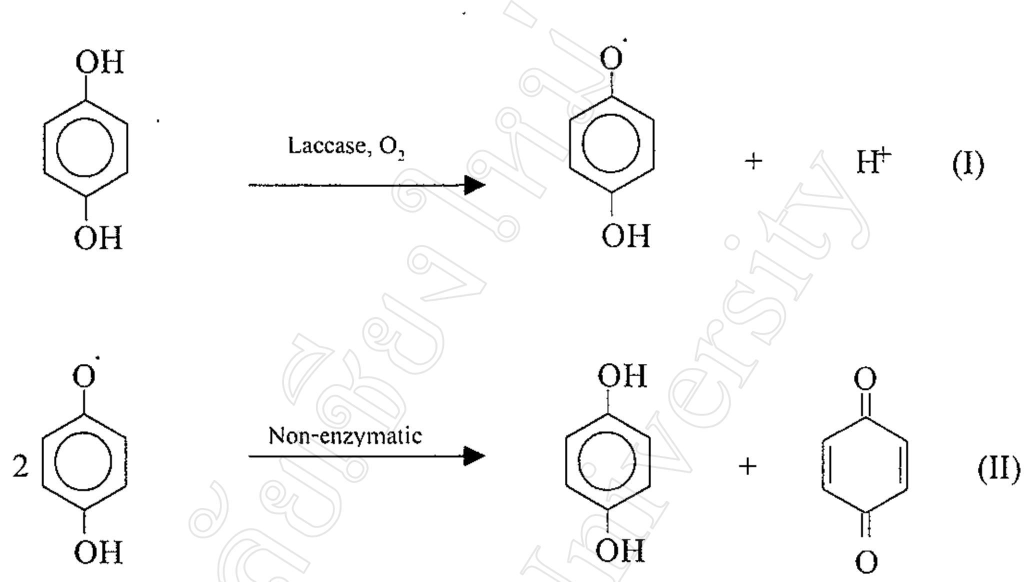


Fig. 1.11 Oxidation of hydroquinone by laccase to give a phenoxy radical which proportionate into hydroquinone and *p*-benzoquinone (Harvey *et al.*, 1986).

Most of fungi produce both intra- and extracellular enzymes. Some compounds, including the substituted phenol, amines, and benzoic acid can induce the synthesis of laccase by some fungi (Sannia *et al.*, 1986). The physiological function of fungal laccase is thought to be primary relation to the role of laccase in lignin decomposition. The major function of laccase may be not the oxidation of lignin structure, but also its influence on polymerization of their oxidation products (Geiger,1986).

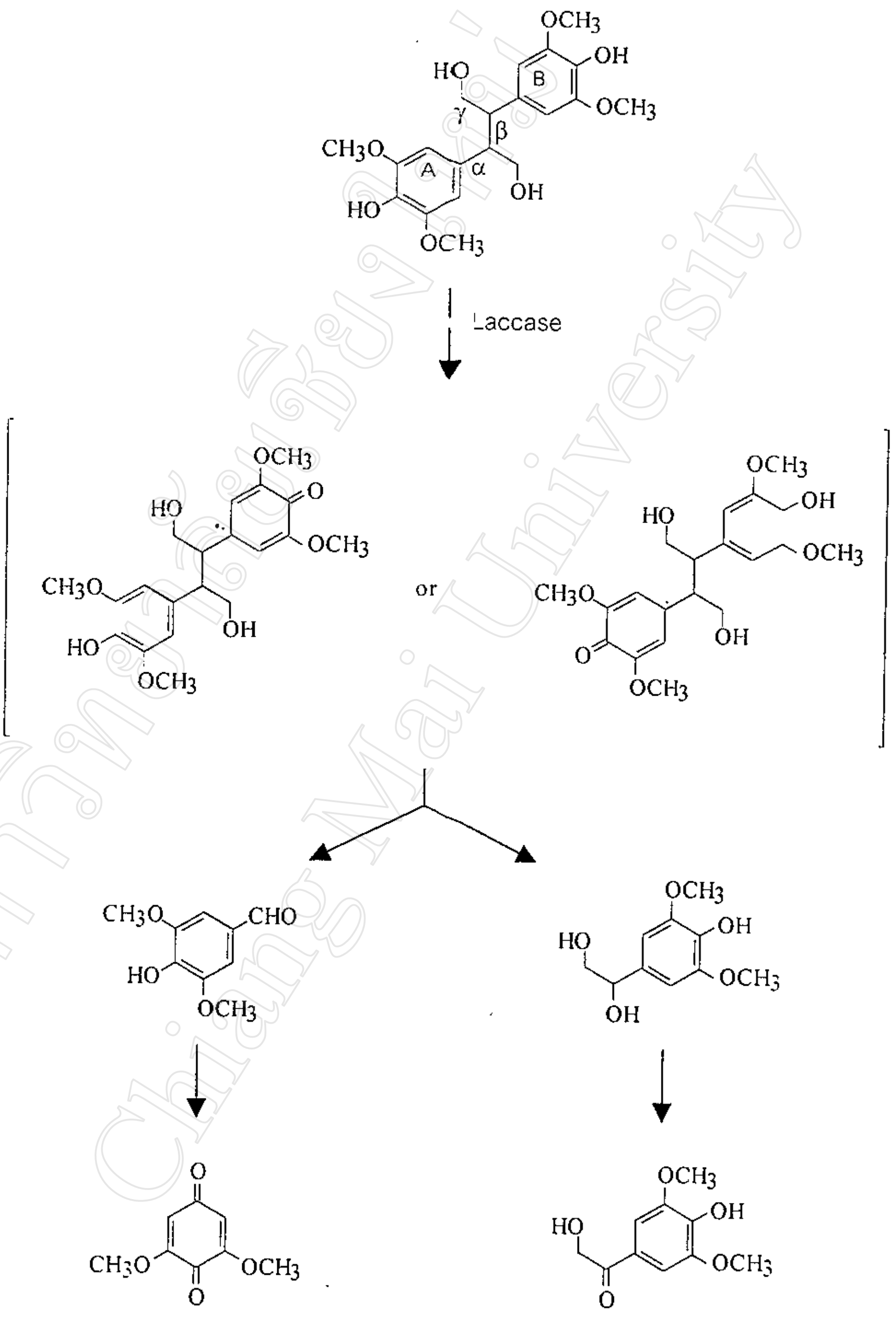


Fig. 1.12 Laccase mechanism acts on a phenolic *β*-1 syringyl lignin substructure by laccase from *C. versicolor* (Kawai *et al.*, 1987).

The laccase enzyme contains atoms of copper in central molecule (Fig. 1.13). It requires oxygen as a cosubstrate for oxidation-reduction of lignin subunit. The treatment of kraft pulps with laccase does not only affect on any degradation of lignin, but its structure is changed or repolymerized. Most laccase producing fungi belong to basidiomycetes, the best natural lignin degrader. The ability of laccase to remove brown color of lignin is a promising alternative way to replace or reduce the use of chlorinated organic compounds in kraft pulp and industrially paper manufacture (Kuram and Nicell, 1997).

Fig. 1.13 Three dimentional structure of a copper amine oxidase (laccase) showing the two monomers of the homodimer color coded in blue and yellow. The large central domains, that are lighter in colour, contain the active sites and are linked by long beta loops from the other monomer. The copper atoms at the active sites are shown as small red spheres (Kawai *et al.*, 1987).

Role of Laccase-mediator system on the paper pulp bleaching

In the past decade, enzymes have made an impact in the industrial bleaching of kraft pulp for the manufacture of paper. Most of the earlier work focused on the use of xylanase to reduce the amounts of chlorine-containing chemicals required to achieve target pulp brightness. Recent success has been achieved for oxidative enzyme at the laboratory scale. In particular, laccase has been found to reduce the kappa number and enhance the bleaching of kraft pulp when used in the presence of a chemical mediator and oxygen (Bourbonnais and Paice, 1992). The implementation of this enzyme process has been restricted by the high cost of the mediator (Bourbonnais *et al.*, 1997).

Because the kappa number is a measure of residual lignin in pulp, its declination after pulp treatment with the laccase-mediator system (LMS) suggests that lignification has occurred. Like other enzyme system, LMS may be highly selective and yield of pulp delignification with little carbohydrate loss or damage (Wong *et al.,* 1999).

The use of LMS for pulp bleaching has increasingly gained attention since the initial discovery. The nonphenolic lignin model compound can be oxidized and pulp delignified by laccase from *Trametes versicolor* in the presence of 2,2’-azono-di(3-ethylbenzothialozin-6-sulfuric acid) (ABTS) (Wong *et al.,* 1999). The interaction between laccase and ABTS affect on residual lignin in kraft pulp was shown in Fig. 1.14. Many reports suggested that the interaction of laccase and ABTS could effectively demethylate and delignify kraft pulp. ABTS formed a stable radical cations following the oxidation. It seems that the oxidized ABTS must function as a diffusible electron carrier because laccase is a large molecule (M_w~70,000). Therefore, it cannot enter the secondary wall to contact the lignin substrate directly (Fig. 1.15). Recently, over 50% delignification of kraft pulp has been reported with laccase and another mediator, 1-hydroxybenzotriazole (Eriksson *et al.*, 1990), or by repeated treatment with laccase/ABTS followed by alkali extraction (Bourbonnais and Price, 1996). These observations have excited considerable interest in enzyme-catalyzed oxidative bleaching of kraft pulps. Even though ABTS is a highly efficient mediator for pulp delignification by laccase, a commercial application does not seem to be feasible in the near future because it is estimated that, even if manufactured in bulk, its price would be too high for cost effective use in industrial pulp bleaching. In further study, to investigate the nature of the chemical interactions between laccase, ABTS or other mediators and lignin. The objective is to design and develop a potential and less cost system for pulp delignification with oxidative enzymes and apply this concept to a commercial application.

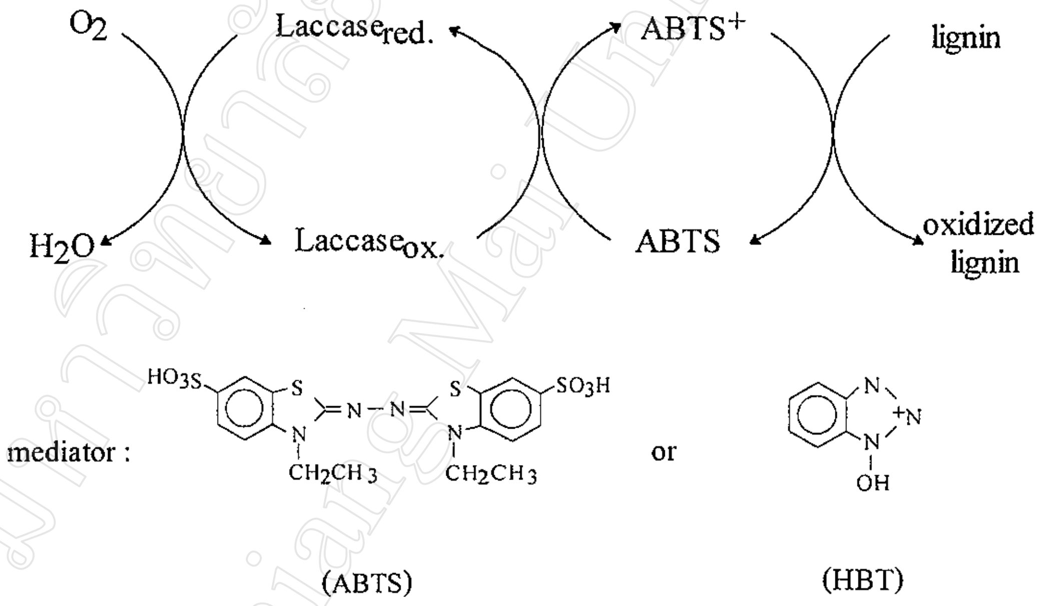


Fig.1.14 Schematic representation of lignin degradation by laccase and mediator system. Structure of two mediators used in LMS for kraft pulp bleaching (Wong *et al.*, 1999).

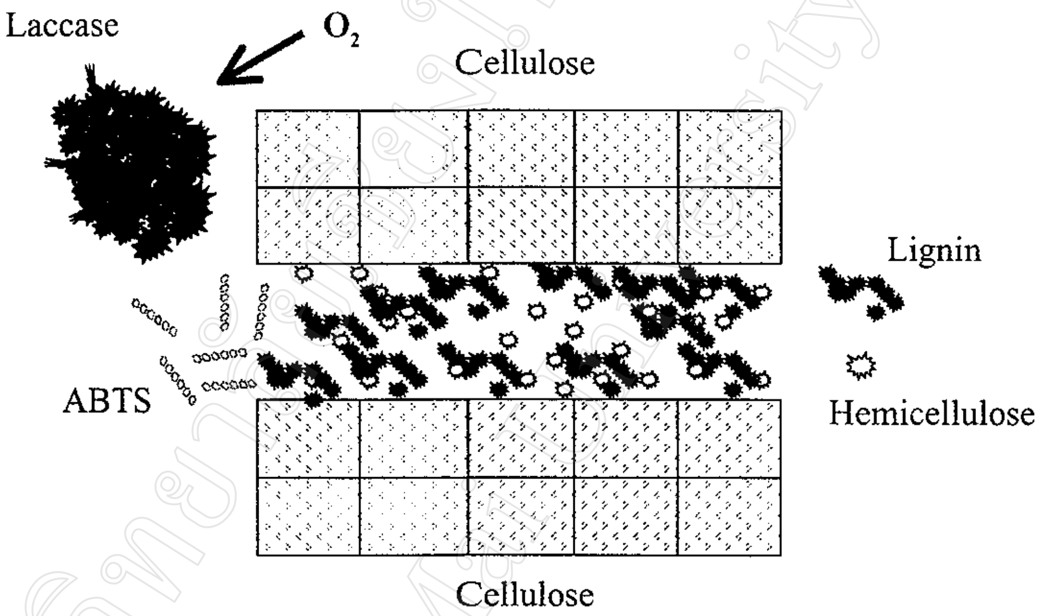


Fig. 1.15 Computer simulation of a cross-section through the secondary wall of an unbleached kraft fiber in proportional comparison with putative images of two *T. versicolor* enzymes and their known mediators. This figure suggests that diffusion of mediators (but not enzymes) in the secondary wall is easily possible (Paice *et al.*, 1993).

Obstacle to implementing bleaching with oxidative enzymes

In 1995, Paice *et al.* observed delignifying activity by laccase and MnPs from *Trametes versicolor* under certain conditions. However, several obstacles remain to be overcome before these oxidizing enzymes can be applied on the large scale:

1. **Availability of enzymes.** Neither enzyme is available in sufficient quantity for mills and scale up of enzyme production from fungal culture may be costly. Cloning of gene for both enzymes has been reported (Mayfield *et al.*, 1994), and may provide an alternative production route.
2. **Cost of chemicals requirement.** The laccase mediators, ABTS and HBT, are expensive, and alternative are less effective because it spent more time when compare with xylanase. Chelating agents for Mn (II) in the MnP reaction may also be a significant cost item.
3. **Enzyme stability.** MnP (and LiP) require H_2O_2 but are inactivated by about 0.1 mM H_2O_2. Even when H_2O_2 is kept below 0.1 mM, MnP becomes inactive relatively rapidly. It has no report to explain this matter clearly yet.

Experiments with xylanase, lipase, and amylase have shown that enzyme can be applied successfully in a mill situation (Paice *et al.*, 1995). Thus, the oxidative enzymes, which can be regarded as the catalysts for oxygen and H_2O_2 driver in delignification, may also find a place in the bleached plant in the coming years.

Application of white rot fungi on pulp and paper industry

Kraft pulping is the dominant process for the production of chemical bleached pulps. The current production of kraft pulp amounts to 60 million tons per year (Kramer, 1992). Conventional kraft pulp bleaching is based on alkali extraction and chlorination of the pulp as described previously. The use of chlorine-based bleaching chemicals results in effluents containing high level of chlorinated organic compounds, which are known to be toxic. The growing public concern over the negative ecological effects of organochlorines has motivated research into the development of new technologies directly to reduce or eliminate the use of chlorine-based chemicals in pulp bleaching. The use of biotechnology in the bleaching of kraft pulp offers good representatives for the development of total chlorine free bleaching sequences. Two main biotechnological approaches have been designed for pulp bleaching. The first is based on the use of xylanase to remove hemicellulose deposits from kraft pulps in order to enhance the subsequent alkali extractability of lignin. Pulp bleaching with xylanase is already being used at kraft mills to facilitate a low dosage of bleaching chemicals (Viikari *et al.*, 1994). The second approach under consideration is the use of white rot fungi or their ligninolytic enzymes to selectively oxidize lignin as the main bleaching step (Reid and Paice, 1994). Although the use of white rot fungi in biobleaching has not yet reached to industrial scale application, continued research efforts are useful since biological removal of lignin is expected to largely substitute bleaching chemicals.

Therefore, the basic knowledge involves in biotechnology has been used in the pulp and paper industry such as wastewater treatment, the control of slime and deposit on paper machines can be considered as an aspect of biotechnology. However, under the past several years, the biotechnologists have found specific applications for microorganism or its enzymes in the pulp and paper production within biotechnological trend.

The process of paper production contained two main parts, in brief, it starts changing wood chips to pulps and then pulp is changed to be paper. The wood pulping is the first step in production of paper from wood chips, pulp is produced from wood either by chemical delignification, mechanical separation of fibers or combination of chemical and mechanical methods, usually mechanical pulping method is used increasingly more than chemical method. The main disadvantages of mechanical pulping are high energy consumption and produces low strength and low brightness stability of mechanical pulping which lead to be low quality of papers. Thus the solution in this problem is attempts to reduce the high energy requirement and to increase property of paper.

Base on the basic knowledge, white rot fungi can reduce the darkness of wood to brightness under natural conditions. From this, the use of this microorganism for pretreatment of wood chips prior to mechanical or chemical pulping which is called biopulping, is a very promising alternative method to solve such problems. The aim of this project is to replace or reduce the chemical usage in pretreatment of wood in mechanical pulping, reduce energy demand and increase paper strength properties. Biopulping is intended to reduce the use of chemicals, to increase the cooking capacity, and to increase delignification efficiency within a direct energy saving for pulping (Kirk *et al.*, 1994). Moreover, it was assumed that fungal pretreatment would have less impact on environment than chemical using. Based on the practical and economical possibility study of biopulping, a chip pile-based system is designed in Fig. 1.16.

In the pulp and paper industry, pulping of wood was done by the large consumptions of energy (mechanical pulping) and chemicals (chemical pulping). The process involves in high temperature and pressure and extreme pH values. Fungal pretreatment of wood chips or pulp, causes delignification, saves energy, and reduce chemical consumption in mechanical and chemical pulping (Eriksson, 1985; Eriksson and Kirk, 1985). Base on the improvement in paper strength properties and potential energy saving by white rot fungi on biomechanical pulping given in Table 1.12, indicate that the possible application of white rot fungi on biopulping is nearly feasible.

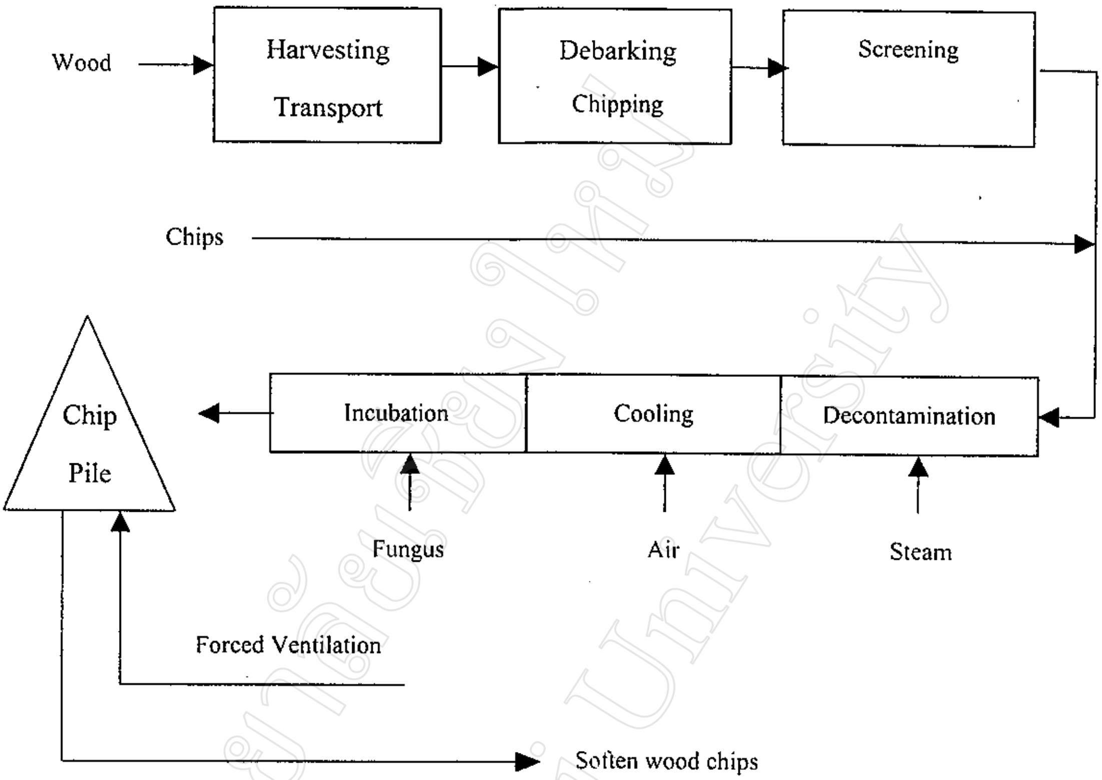


Fig. 1.16 Schematic of proposed biopulping process. (Bajpai *et al.*, 1999).

Table 1.12 Energy saving from biomechanical pulping of loblolly pine chips with different white rot fungi (4-week incubation)

Fungus	Energy savings (%)
Phanerochaete chrysosporium	14
Hyphodontia setulosa	26
Phlebia brevispora	28
Pklebia subserialis	32
Phlebia tremellosa	36
Ceriporiopsis subvermispora	42

Base on data from Bajpai *et al.* (1999).

White rot fungi have greatest potential for biotechnological applications because they do not only attack lignin into the depth of wood chips, but also produce the whole set of enzymes, which are necessary for lignin degradation in the biobleaching process.

White rot fungi which extensively attack lignin in wood are able to bleach kraft pulps. Kirk and Yung (1979) were the first to attempt to bleach pulp with microorganism. They observed that *Phanerochaete chrysosporium* and some other fungi could show lower kappa number of unbleached softwood kraft pulp by up to 75% and lead to reduce the requirement of chlorine during chemical bleaching. Table 1.13 shows the achievement of chemical saving in wood kraft pulp bleaching with *Coriolus versicolor* IZU-154.

Table 1.13 Bleaching conditions and optical properties of conventionally bleached and biobleached commercial SWKP

	Dosage (% on pulp)						Brightness (% ISO)			
Bleaching sequence	C	E_1	D_1	E_2	D_2	As effective chlorine	Before aging	After aging	PC number	Yield (%)
Conventional process	9.0	6.4	1.0	0.5	0.5	12.95	84.2	82.4	0.38	91.4
Biobleaching process	2.4	1.7	0.4	...	...	3.45	84.6	83.4	0.25	91.4

Based on data from Futika *et al.* (1993)

Since xylanase was found to improve the bleaching ability of kraft pulp (Viikari *et al.*, 1986) and are now being produced for the pulp industry by several companies around the world (Viikari *et al.*, 1994). Many mills worldwide have experience in using xylanase for prebleaching process and found that the result up to 20%-25% saving in chemical bleaching with reduction of pollution emissions. Comparison to the different efficiency of laccase and xylanase which is indirectly attacked lignin is shown in Table 1.14. Laccase has more effective on pulp bleaching than xylanase, although, there is no any degradtion of lignin by this enzyme.

Table 1.14 Differences in xylanase and laccase/mediator treatment

	Xylanase	Laccase/mediator
1	No or very poor kappa number reduction	Very good kappa number reduction
2	Moderate bleaching effect	Good bleaching effect
3	Saving of bleaching chemicals	Saving of bleaching chemicals
4	-	TCF pulp production possible

Note: Based on data from Bajpai *et al.* (1999)

An example of the efficiency of fungal treatment chlorinayed compounds from the effluent treated by *Rhizopus oryzae* in India is shown in Table 1.15.

Table 1.15 Effect of *Rhizopus oryzae* treatment on chlorophenols and chloroaldehydes in extraction-stage effluent

Compounds	Concentration (mg/l) in: Untreated effluent	Concentration (mg/l) in: Treated effluent	Removal (%)
2-Chlorophenol	4.98	0	100
4-Chlorophenol	0.1265	0.0076	94 1.4
2,6-Dichlorophenol	0.1130	0	100
5-Chloroguaiacol	9.60	0	100
4-Chlorocatechol	0.2380	0	100
4,6-Dichloroguaiacol	$3.79\text{x}10^{-3}$	0	100
4,5- Dichloroguaiacol	$1.16\text{x}10^{-3}$	$0.62\ \text{x}10^{-3}$	53 ± 2.3
3,5-Dichlorocatechol	0.0154	0	100
3,4,6-Trichloroguaiacol	$3.9\ \text{x}10^{-3}$	$0.2\ \text{x}10^{-3}$	95 ± 1.1
4,5- Dichlorocatechol	0.014	0	100
3,4,5- Trichloroguaiacol	$5.385\ \text{x}10^{-3}$	0	100
4,5,6- Trichloroguaiacol	0.01466	0.004	72 ± 1.6
Tetrachloroguaiacol	$2.6732\ \text{x}10^{-3}$	$1.12\ \text{x}10^{-3}$	58 ± 2.2
2-Chlorosyringaldehyde	0.065	0.033	51 ± 1.6
Trichlorosyringaldehyde	$8.9\ \text{x}10^{-3}$	0	100
2,6-Dichlorosyringaldehyde	0.029	0	100

Based on data from Nagarathnamma and Bajpai (1999)

From an ability of the white rot fungi, leads to be an opportunity to use them for direct attack lignin and use their potential enzymes in kraft pulps and wastewater treatment. Some of promising and possible results have been recently obtained which were shown as follows;

Buchert and co-workers (1993) explained the capability of xylanase and mannanase from *Trichoderma reesei* for using in paper pulp biobleaching. In the same year, Kantelinen *et al.* (1993) discussed the potential of lignin peroxidase and laccase from *Phlebia radiata* for applications in lignin degradation. Both enzymes from *Phlebia radiata* were able to bleach *Pine* pulp as well as enzymes from *P. chrysosporium,* which showed the brightness up to 42.2% ISO while the enzymes from *P. chrysosporium* could show the brightness around 44.8% ISO. Furthermore, the use of both lignin degrading enzymes and hemicellulases could show the potential efficiency in kraft pulp bleaching.

In 1995, Katagira *et al.* discovered the role of Manganese peroxidase, lignin peroxidase, and laccase in pulp biobleaching. They had examined the relationship between the brightness of unbleached kraft pulp and activity of these enzymes production on solid state fermentation by *Phanerocheate chrysosporium* and *Trametes versicolor*. Manganese peroxidase was the most important enzyme among the whole enzymes from white rot fungi on biobleaching of unbleached kraft pulp by two strains of fungi.

In 1995, Bourbonnais *et al.* had focused on lignin oxidation by laccase isoenzymes from *Trametes versicolor* and the role of mediator, ABTS, in kraft lignin depolymerization. From the results, they found that *Trametes versicolor* could produce two laccase isoenzymes (I and II). Although, there were some difference in the oxidation rate of polymeric substrates, their qualitative effects on kraft lignin and residual lignin in kraft pulp were similar. Furthermore, the reduction of kappa number of softwood and hardwood pulp by two isoenzymes were noticeable in only the presence of ABTS.

In 1997, Archibald *et al.* reported for an ability of *Trametes versicolor* on delignification and kraft pulp bleaching. They found that this strain was able to bleach oxygen-delignified and extended-cooking pulp as well as conventional bleaching of hardwood and softwood kraft pulp. There are evidence of pulp biobleaching by two laccases from *Trametes versicolor* but there not of lignin depolymerization. Both laccases were able to delignify kraft pulp only in the presence of mediator, ABTS, because it prevented and reversed the polymerization of kraft pulp lignin by two laccases. For the delignification of kraft pulp by manganese peroxidase from the same culture of *Trametes versicolor*, it required chelators including hydrogen peroxide and manganese ion. In the presence of Mn^{2+}, the activity of secreted manganese peroxidase could be observed, therefore, this ion was necessary for removal of lignin from kraft pulp and pulp brightening by manganese peroxidase uses.

In 1997, Jong *et al.* tested five groups of fungi including white rots, brown rots, litter degrading fungi, sapstrain fungi, and Hypomycetes for biopulping treatment of Douglas mechanical fir pulp. Most strains which showed brightness improvement belong to white rot fungi, although the improvement of pulp strength properties were not so markedly different.

In the same year, Bourbonnais *et al.* (1997) reported for the various mediators for using in kraft pulp biobleaching by laccase from *Trametes versicolor.* They attempted to find the new mediator that was inexpensive, low dosage requirement, and reduced high potential support of kraft pulp bleaching for replacement of ABTS, which was a well-known mediator for laccase activity. From the results, HBT was found to be the most attractive mediator because it reduced the most intensive delignification over 2 hrs but its oxygen uptake with laccase was slower than ABTS. From these, indicated that ABTS was still the useful mediator and HBT and ABTS were required for kraft pulp bleaching by laccase.

In 1998, *Trametes versicolor* was used for decoloration of bagasse-base paper mill effluent which contained 2840 point color units and pH 7.0-8.0. The fungus was able to maximally decolorize color to be around 60% and was achieved on the fourth day of culture in the effluent inoculated with the strain after addition of (w/v) 0.175% ammonium tatrate and 1.0% glucose as cosubstrates.

In 1999, Nagarathnamma *et al.* investigated on biological treatability of extraction stage effluent in term of decoloration, dechlorination, and detoxification by *Ceriporiopsis subvermispora* CZ-3. The strain was able to remove up to 90% color, 45% COD, 62% lignin, 32% AOX, and 36% EOX in 48 hr at 30-35°C and pH 4.0-4.5 in the presence of 0.1% (w/v) glucose as cosubstrate.

In the same year, Swamy and Ramsay (1999) discovered the effects of glucose and ammonium concentration on sequential decoloration of textile dyes by *Trametes versicolor*. They discovered that ability of decoloration was increased when increase glucose concentration. Glucose was consumed at a steady rate and was completely depleted by the fifteenth day. In the case of ammonium concentration, the decoloration rate was inhibited when ammonium tartrate concentration was increased from 0.086 to 0.86 g/l. From the result they suggested that rate of decoloration of textile dyes by *Trametes versicolor* reversibly depend on ammonium concentration. but it directly depended on glucose concentration.

In 1999, Moreira *et al.* compared the Mn^{2+} requirement for biobleaching of eucalyptus oxygen-delignified kraft pulp (OKP) by five white rot fungi including *Trametes versicolor, Phanerocheate sordida, Phlebia radiata, Stereum hirsutum,* and *Bjerkandera* sp. strain BOS55. All strains were able to produce MnP and provided extensive bleaching of OKP when 33 μM of Mn was added into the culture. *Bjerkandera* sp strain BOS55 was the only strain that exhibited MnP production and biobleaching activity of EDTA extracted OKP in the absence of Mn. The results supported the suggestion that MnP was the most effective enzyme in pulp biobleaching when compared with other strains.

In 2001, Moreira *et al.* used the strains *Bjerkandera* sp. strain BOS55, which caused high level of kraft pulp bleaching for MnP production. The enzyme was used for eucalyptus oxygen-delignified kraft pulp (OKP) bleaching. The optimal concentration of Mn and H_2O_2 dosage were found to be 100-500 μM and 0.017 μmol/min ml, respectively. Organic acid buffer was optimal for MnP activity and MnP was applied 60 unit/gram of pulp could decrease significantly kappa number to be around 11-13%. However, excess of H_2O_2 caused to inactivate MnP activity during the incubation.

From those possibilities of white rot fungi applications, since 1981, the International conferences on biotechnology in the pulp and paper industry have been organized every 3 years to evaluate the development in biotechnological field. The current developmental stage of various biotechnological approaches for use in pulp and paper industry is shown in Table 1.16. Although many applications are still in the R&D stage, in the near future, the applications of biotechnology are still expected to be the potential replacement of traditional bleaching process in the pulp and paper manufacturing.

From those possibilities of application from white rot fungi on environmental protection lead to increasingly focus on laccase ability for study of its applications in pulp and paper industry. Therefore the main purposes of this thesis are to seek the best candidate of laccase producer, to induce enzyme production, to purify laccase prior to characterize its properties, and to investigate this purified enzyme in the preliminary biobleaching of paper pulp.

Table 1.16 Current status of biotechnology in the pulp and paper industry

Process	Status
Biological depithing	Commercial scale
Removal of pitch in pulp by enzymes	Commercial scale
Xylanases for pulp bleaching	Commercial scale
Removal of pitch in wood chips by microorganism	Commercial scale
Improvement of pulp drainage by enzymes	Commercial scale
Enzymatic deinking	Commercial scale
Pulp bleaching by laccase mediator system	Pilot scale
Biomechanical pulping	Pilot scale
Purification of bleach plant effluents	Pilot scale
Production of dissolving pulp	Laboratory scale
Use of enzymes for debarking	Laboratory scale
Use of enzymes for retting of flex fiber	Laboratory scale

Based on data from Bajpai *et al.*, (1999)

Evolution of paper pulp biobleaching by microorganisms

In 1965, Kent Kirk from the Forest Product Laboratory, the United States Department of Agriculture and Kart-Eric Eriksson, a Swedish from the Swedish Forest Product Research Laboratory, studied the role of white rot fungi on the removal of lignin from kraft pulp. *Phanerocheate chrysosporium* was the first representative of white rot fungi, which has been used as model to study for pulp biobleaching. This strain was directly cultivated on kraft pulp to compare with the use of chemical bleaching by using 27% chlorine as lignin degrader. The experiment was succeeded in 1979, but there was no report about the utilization of white rot fungi for application in paper pulp biobleaching in industrial scale yet (Kirk, 1979).

In 1983, Tein and Kirk evaluated the function and mechanism of the whole enzymes from white rot fungi. Unfortunately, they were unable to produce and use the lignin degrading enzymes from the fungi directly. Afterward, they used hydrolyzed xylan and hemicellulose, which was one of the main component in wood. Xylanase was able to hydrolyze xylan to simple structures made it easier to remove lignin from kraft pulp.

Enari *et al.* (1983) had studied for production of hemicellulases and tried to reduce amount of cellulases. They tried to separate cellolases from hemicellulases. Hemicellulases alone was able to show the potential bleaching of kraft pulp better than the mixture of hemicellulase-cellulases.

In 1987, Ramachadra *et al.* reported the first lignin degrading enzymes from *Streptomyces* spp, *Streptomyces viridosporus* T7A and two mutants, UV irradiation mutant (T7A-81) and protoplast fusion recombinant (SR-10), with enhanced abilities to produce a water soluble degradation intermediate, an acid-precipitaton polymeric lignin (APPL). They were cultivated on dampened corn stover in solid-state fermentation. Two mutants showed higher and longer continued peroxidase, esterase, and endoglucanase activities than the wild type strain T7A. Three endoglucanase, three esterase, and four peroxidase isoenzymes, which were induced by lignocellulose and APPL were discovered. The enzyme properties were similar to ligninase from fungi.

In 1989, the Enso Gutzeit Company attempted to use the enzymes for pulp biobleaching in practical field at Uimaharja factory, Japan. There was no effect on environment thus the competition of enzymatic paper pulp bleaching have been occurred and wood degrading enzymes have been concentrated dramatically (Higuchi, 1993).

In 1989, Adhi *et al.* studied the lignocellulose degrading enzyme from two strains of *Streptomyces* in agitated submerged culture. The two strains, *Streptomyces viridosporus* T7A and *Streptomyces badius* 252 were grown on 1 to 2% (w/v) slurry culture with mineral salt solution containing 0.6% (w/v) yeast extract and 100/200 mesh ground and extracted corn lignocellulose at 37°C. *S. badius* 252 was capable to produce lignin peroxidase with the concentration of three folds higher than *S. viridosporus* T7A after 9-10 d of incubation, although its endoglucanase and xylanase concentration were lower than those produced from *S. viridosporus* T7A. *S. badius* 252 produced four isoenzymes of peroxidase but only lignin peroxidase showed high activity when detected by polyacrylamide gel analysis.

In 1993, Kantelinen *et al.* studied the lignin peroxidase and laccase production from *Phlebia radiata*. The culture contained either wood powder lignin (WPL) or black liquor lignin (BLL) for induction of enzymes. It was found that WPL was easily degraded by *P. radiata* and was slightly changed in the molecular mass distribution by laccase. On the other hand, BLL was very resistant even to fungal attack, although some changes were detected. Bleachability of kraft pine pulp was treated and found that only laccase could improve this pulp after hemicellulase treatment. From these results, they suggested that hemicellulase treatment could increase both lignin extractability and the accessibility of lignin by lignin modifying enzymes.

Iimori *et al.* (1994) isolated *P. chrysosporium* SKB-1152, which showed capability to increase the brightness of oxygen delignified kraft pulp from 51.15% to be 80% after 7 d of incubation. In the same year, Leontievsky and his co-worker (Leontievsky *et al.*, 1994) had cultivated white rot fungi *Panus tigrinus*, which increased the productivity of manganese peroxidase in liquid medium without lignin peroxidase and laccase production. Moreover, the addition of Tween-80 into basal medium for growth enhancement and enzymes production could increase the efficiency of manganese peroxidase activity up to 2.5-3.0 folds compared with the control. The addition of 3-methylbenzyl alcohol led to induce production of manganese peroxidase and could increase the potential activity of enzyme up to 2-4 folds.

In 1995, Mester *et al.* studied on manganese regulation of veratryl alcohol in white rot fungi and its indirect effect on lignin peroxidase. Many white rot fungi were able to produce *de novo* veratryl alcohol, which was a cofactor in lignin degradation, lignin model compounds, and xenobiotic pollutants by lignin peroxidase. Manganese had strong influence on endogenous veratryl alcohol level in the culture fluid of N-deregulated and N-regulated with white rot fungi, *Bjerkandera* sp. strain BOS55 and *Phanerocheate chrysosporium* BKM-F-1767, respectively. The lignin peroxidase production was enhanced by the increased production of veratryl alcohol. Veratryl alcohol protected lignin peroxidase from being inactivated by physiological level of hydrogen peroxide. This confirmed that lignin peroxidase was regulated by Mn^{2+}.

Barbosa *et al.* (1996) used veratryl alcohol to induce laccase production from ascomycete isolated from diverse environments in Western Australia. Poly R-478 was used as indicator for screening of isolates and used glucose as a carbon source in liquid medium and used ryegrass seed as a carbon source in solid culture for laccase production. Isolate of *Botryosphaeria* sp., which was isolated from eucalyptus tree, increased the activity of laccase in liquid medium in the presence of 3,4-dimethoxybenzyl (veratryl) alcohol (40 mM). This activity was greater than the addition of glucose only by 115 folds.

Buswell and his colleaques (1996) had studied lignin degrading enzymes from three edible mushrooms, *Lentinular edudes, Volvariella volvacea,* and *Pleurotus sajor-caju.* All of them were able to produce lignin peroxidase and cellulase. In particular, *L. edodes* was able to produce laccase and manganese peroxidase when cultivated on a solid culture. The manganese peroxidase activity of *L. edodes* was 300 unit/ml at the cultivation time at 12 weeks. Furthermore, the enzyme production was dependent on nitrogen concentration.

In 1996, Asiegbu *et al.* studied the influence of ferulic acid on enzymes activity to degrade lignocellulose. Five fungi, *Trichoderma hazianum, Chaetomium celluloticum, Phanerocheate chrysosporium, Trametes versicolor*, and *Pleurotus sajor caju* were severely repressed by 5 g/l of ferulic acid, which was a plant cell wall phenolic. 0.5 g/l of the compound could stimulate the growth of *T. versicolor* and *P. sajor-caju.*

In 1997, Kumaran *et al.* used *Sajo hampus*, the fibrous pith residue from starch extraction from sajo palm as a substrate for laccase production by solid substrate fermentation with *Pleurotus sajor caju,* an edible mushroom. The fungus exhibited highest laccase activity after sixth day of incubation. Approximately 17.7 units/g laccase activity was found in 4 week-old inoculum, which was more than 12 folds compared to 2 week-old inoculum. Addition of 0.2 mM vanillin or ferulic acid could doubly increase laccase activity when compared with the control. The cellulose to lignin ratio was increased significantly after 12 d of SSF from 2.74 to 3.3 when 0.2 mM either vanillin or ferulic acid was added to the substrate.

In 1997, Moveira *et al.* found *Bjerkandera* sp. BOS55, which capabled to increase the brightness of oxygen oxidative pulp to be around 82% ISO. This isolate capabled to induce enzyme production when cultivated on nitrogen limited medium containing 22 mM of NH_4^+.

In 1999, Pointing and workers reported the simple and rapid method for screening of lignin degrading fungi on 0.02% (w/v) Poly R-478 agar by using halo formation as indicator.

Palmieri *et al.* (1999) found the effect of copper induction on laccase isoenzyme production in *Pleurotus ostreatus*, an edible mushroom. 150 µM copper sulfate was able to induce laccase isoenzyme gene transcription, which led to increase enzyme production when compared with control without copper sulfate.