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Enzymes of White-rot Fungi Cooperate in Biodeterioration of Lignin Barrier*1

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목질리그닌의 생물학적 분해시 백색 부후균류 효소들의 상호작용*1

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요 약

목재를 분해시키는 담자균류들은 목재 및 목질복합체에 쉽사리 침투하여 복잡한 리그노셀룰로오스 복합체를 분해시킨다. 이러한 분해에는 많은 효소시스템들이 복합적으로 작용하면서 상호 협동하는것으로 보고되고 있다. 지금까지 알려진 효소들은 통상 3개의 그룹으로 나눌 수 있는데, 그 하나는 목재성분을 직접적으로 공격하는 효소균들, 예를 들면 cellulase complex, laccase(LAC), lignin peroxidase(LIP), horse-radish peroxidase(HRP), manganese-independent peroxidase(MIP) 및 protocatechuate 3,4-dioxygenase(PCD) 등이 있고, 두번째 그룹으로서 manganese- dependent peroxidase(MnP), aryl alcohol oxidase(AAO), 및 glyoxal oxidase(GLO) 등인데, 이들 효소들은 목질을 직접적으로 공격하지 않고 제1그룹의 효소들과 협동하여 작용하는 것으로 알려지고 있다. 제3그룹의 효소들은 glucose oxidase(GOD) 및 cellobiose : quinone oxidoreductase(CBQ)로서 feedback type의 효소들로서 목재고분자의 분해시 대사의 고리를 결합시켜 주는 매우 중요한 기능을 하는 효소군들이다. 그러나 이 이외에도 다른 분해기구가 밝혀지고 있으며, 기타 효소들에 의한 리그노셀룰로오스의 분해반응기구의 해명에는 상당한 시간이 걸릴것으로 사료된다.

Keywords : White-rot fungi, basidiomycetes, lignocellulose, laccase, cellulase, biotransformation, ligninase

1. INTRODUCTION

1.1 What means the ligninocellulose complex and why its structure is so durable?

The ligninocellulose is a compact, almost crystalline complex. Polysaccharide components in microfibers are densely packed in layers of lignin, which protects them from the activity of hydrolytic enzymes and other external factors, and serves as a stabilizer of the complex structure.

This structure endows plants with the neces-

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sary stiffness. It serves therefore in plants as a kind of concrete block with metal rods inside. How durable this material can be is seen from the fact that if the Chicago Sears Tower, the highest building in the world (much over a hundred stories) had had a wooden pile foundation, it would have been 10 times as light as that of traditional concrete and would have required only 20 piles (with the diameter of no more than 1 meter) to hold the construction. In fact, any high-rise block of flats built of wood could be 10 times as high as a concrete one the same circumference. Where such durability comes from? It comes from a compact structure of the ligninocellulose complex. One can discern an analogy between metal rod inside a prefabricated elements and cellulose fibers in lignin.

1.2 What ligninocellulose means in the world ?

The sources of ligninocellulose that occur in various forms in the nature are so vast that they can only be comparable to those of water. The world production of cellulose (the most abundant constituent of the lignocellulose complex) by the process of photosynthesis amounts to 24 tons per capita a year. Taking into consideration of the fact that it is an organic substance it seems that only one step forward to utilize it as a source of food would suffice for the problem of hunger disappear. This (apparently difficult) step consists in transforming the substance in question into easily available products. But how should one do it? Especially how to degrade a lignin barrier protecting carbohydrates in lignin-containing plants. In this paper authors would like to present some results, conceptions and hypothesis of how to do it using wood rotting fungi and their enzymes. But, by the first of all authors would like to introduce some knowledge on lignin which consists a natural barrier against to the hydrolytic enzymes. These enzymes are active only on carbohydrate constituent of complex. They can not destroy lignin at all.

1.3 Why lignin was named "lignin", and how look its structure, distribution and organization in the cell walls ?

The term "lignin" is derived from Latin word "lignum", meaning wood. It was introduced by Anselme Payen in 1838, to represent cellulose-encrusting substances in the lignified plant cell walls. It is a polymer composed of phenylpropane subunits joined by strong carbon-carbon and alkyl-aryl ether bonds as well as hydrogen bonds. Finally, the three dimensional network structure of lignin forms a very stable monolith. Carbon atoms of the subunits both in the aromatic ring and in the side chain can be substituted by means of methoxyl, carboxyl and carbonyl groups. It creates additional possibilities of hydrogen bonds between some fragments of a macromolecule and endows lignin with almost proverbial chemical stability.

Being widely distributed in nature, lignin is the second (besides cellulose) most abundant natural polymer in the biosphere and the most abundant aromatic material constituting about 40% of the solar energy in plants. During phylogenetic development of plants lignin had appeared first in *Pteridophytae*. Presently, it is found in higher plants, mosses, and plants of lower taxonomic ranking; neither algae nor liverworts contain this substance. The highest proportions of lignin occur in dead sclerenchymatous cells (in the spaces of intercellulose microfibrils in primary and secondary walls) and is vascular parts of the conductive tissue in middle lamellae as a cementing component which connects cells and hardens the cell walls of xylem tissue. In wood, most of the lignin (ca. 80%) is found within the cell walls, where is interspersed with the hemicelluloses, forming a matrix surrounding cellulose microfibrils. As the plant grows older, the percentage of lignin rises, up to 29% in conifers and 26% in broad-leaved species on a dry matter basis.

1.4 How fungi can penetrate the wood tissue and how to secrete their enzymes from the hyphae of the mycelium to wood environment ?

The fungi which are responsible for wood degradation usually grow into the region of lignified cells. The so-called soft-rot fungi grow in the secondary walls of wood fibers and there form cylindrical cavities with conical ends. The hyphae of brown-rot and white-rot fungi are localized in the cell lumen. The white-rot fungi penetrate from one cell to another through the openings which occur in the structure of wood or through the channels bored previously in the cell walls. The soft-rot fungi cause softening of a wood surface layer which gives the name to this group of organisms. This process consists in a preliminary transformation of both wood components. The white-rot and (less) brown-rot fungi attack lignin by oxidative cleavage of a side chain of phenylpropane units, removal of methoxyl groups and cleavage of aromatic rings. The group of white-rot fungi is dominant in these processes, among them *Trametes versicolor, Phanerochaete chrysosporium and Phlebia radiata* are well known lignin destructors. An attack of brown-rot fungi is characterized by the gradual diminishing of the thickness of cell walls caused by enzymatic hydrolysis of polysaccharides with only a slight disarrangement of lignin structure.

The mycelial hyphae grow into the lignocellulosic material under the attack with a cone-like ending of a extended cell in the shape of a spindle. This is the top area of a cell where the fastest growth of hyphae can be noted. Beyond this point, there is no particular assimilation of substrates into the interior of the cell. The top most area of the hyphae is functionally and cytologically different from the other segments. One can notice in an electron microscope numerous cytoplasmic vesicles functioning as carriers for the substrates and enzymes necessary for constructing the cell wall as well as the enzyme secreted extracellular. Cytoplasmic vesicles are formed in dictyosomes of the Golgi apparatus located near the endoplasmic reticulum which synthesizes those enzymes and other proteins constituting a part of it (see Fig. 1, I). During the transitory phase (Fig. 1, II) cisternae are formed from the reticulum membrane: they gradually become filled with the material and are later divided into smaller vesicles (Fig. 1, III). From dictyosomes, vesicles move toward the shred top (Fig. 1, IV) where they are penetrate into the area of their own cell wall synthesis or discharge their contents extracellular (Fig. 1, V).

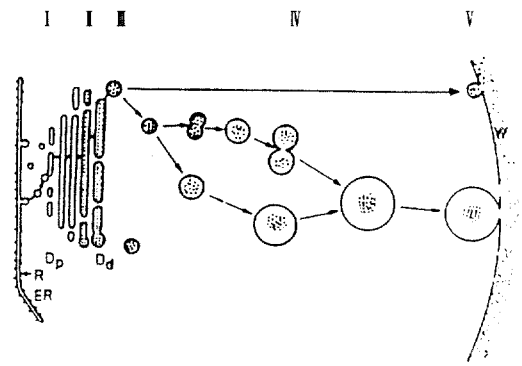

Fig. 1. Diagrammatic interpretation of the probable sequence leading to the expansion of hyphae at the apex. Dd - distal pool of dictyosome; Dp: proximal pool of dictyosome; ER: endoplasmic reticulum; R: ribosome(Grove *et al.*, 1970)

It has been established, that the enzymes secreted into the medium are stable and able to function exrtracellular. It is commonly believed that most of the enzymes transforming ligninocellulose originate in this way.

2. BIODEGRADATION OF WOOD CONSTITUENTS

It is probably mainly an enzymatic process. The transformation of free cellulose or herni-

cellulose into monosaccharides has long been known to be relatively simple. Many cellulolytic and also some ligninolytic fungi make use of a full range of hydrolases able to produce monosaccharides in large quantities from all polysaccharide component of ligninocellulose. However, it is mandatory for these components not to occur in a complex with lignin. Thus, lignin with its barrier against hydrolytic enzymes causes the problem. Among other things, this is why the research on the biotransformation of ligninocellulose complex has been carried out for years. So the main topic of this article will be overview of biotransformation of lignin.

2.1 The piece of history

As early as the beginning of 1930s some attempts were made to delignify wood by means of white-rot fungi. According to Campbell (1930) and, quite independently, Wiertelak (1932) *Trametes versicolor* and *Trametes pini* were the fungi that transformed wood most actively. Their researches were extremely innovative in this field. Several years later a more detailed analysis of the process of delignification of beech sawdust by white-rot fungi as *Polyporus abietinus*, *Stereum rugosum* and *Marasmius scorodonius* was carried out(Fahraeus *et al.*, 1949). But as the analytical methods at that time were poor, the more detailed analysis of the process of delignification was very difficult.

2.2 The first attempts to explain, how do microorganisms degrade the lignified parts of plants

The development of chromatographic methods helped to explain some stages of lignin transformation by a detailed analysis of lignin degradation products. So in 1960s(Fukuzumi, 1960; Ishikawa *et al.*, 1963) appeared first mechanisms of lignin degradation by white-rot fungus, *Poria subacida*, grown on wood meal. These mechanisms however did not show which enzymes and how were able to cause the process of lignin biotransformation and how did lignin barrier be able to be destroyed.

3. WHICH ENZYMES CAN DIRECTLY DEGRADE THE LIGNIN BARRIER ?

Below will be listed and shortly described the enzymes considered as probably the lignin degraders. Their known up to date ability on lignin biopolymer will be also mentioned.

3.1 Laccase (LAC)

Laccase is a multiple form enzyme excreted into medium by mycelial of several Basidiomycetes, Ascomycetes and Deuteromycetes. In some fungi, laccase can be induced by xylidine or methoxyphenolic acid(Bollag & Leonowicz, 1984). The white-rot fungi showing ability on lignin transformation produce the highest amounts of laccase(Leonowicz *et al.*, 1997a). In our very early report we have shown that such a process would be catalyzed by laccase(and peroxidase) through preliminary demethylation of lignin, what is an essential for starting degradative process. After demethylation breaking of etheric bonds which bind lignin subunits is probably spontaneous(non-enzymatic) and takes place as a result of electrophilic effect of ortho-quinone groups on the etheric bond joining two lignin phenylpropane subunits(Leonowicz & Trojanowski, 1965).

This old report by the first time postulated the possible role of laccase and peroxidase in lignin degradation. The earliest confirmation of such conception could be find in the work of Harkin and Obst (1974). They showed that laccase or peroxidase generated a number of phenoxy radicals during the process of 2,4,6-trimethoxyphenol oxidation. Mesomeric forms of the phenoxy radicals are further coupled into dimers and during oxidation into corresponding quinones they undergo demethylation and decompose into monomers(see Fig. 2).

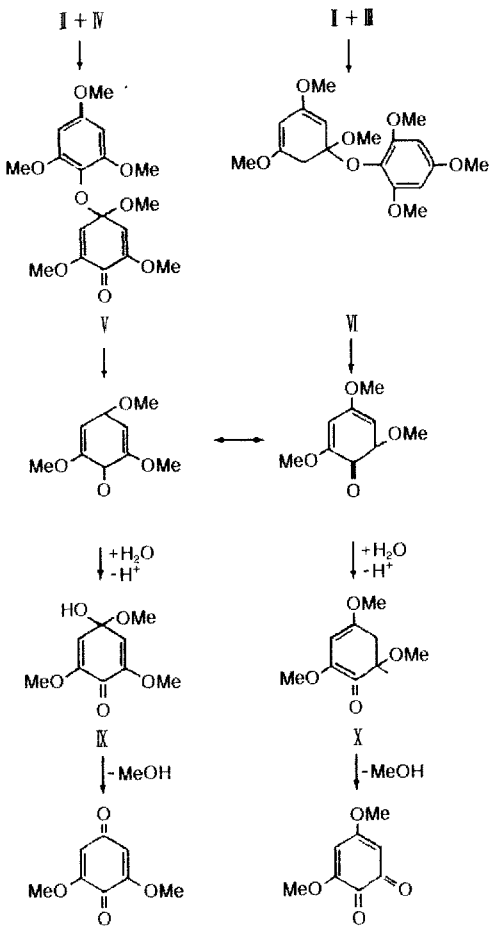

Fig. 2. Transformation of dimers formed from 2, 4, 6-trimetoxyphenol during laccase action. Decomposition of dimers is accompanied by oxidation and demethylation processes(Harkin & Obst, 1974).

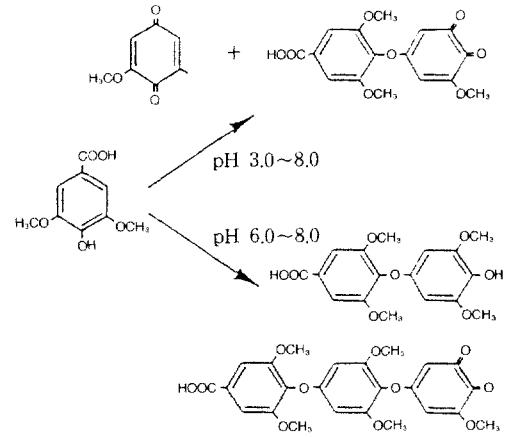

Fig. 3. Oxidation and demethylation of syringic acid by Rhizoctonia praticola and *Trametes versicolor* at different pH values (Leonowicz *et al.*, 1984).

The Harkin and Obst 's report confirmed then the ability of laccase (or peroxidase) to cleave etheric bonds joining aromatic subunits. It would also testify to the role of demethylation and formation of quinone compounds in this process. Subsequent research proved repeatedly the demethylating activity of laccase, e.g. the demethylation of syringic acid (Ishihara & Ishihara, 1976) and the effect of pH on this process (Ishihara, 1983; Leonowicz *et al.*, 1984; see Fig. 3), demethylation of vanillyl glycol(Lundquist & Kristersson, 1985) and vanillic acid (Ander *et al.*, 1983 · 1985). The last authors presented a possible metabolic pathway of vanillic acid through demethylation up to the cleavage of the aromatic ring and ketoacid formation (Ander *et al.*, 1983). Such cleaving of aromatic ring by *Coriolus versicolor* laccase reported also Kawai *et al.*(1988).

The contribution of laccase directly to lignin transformation has been reported by many others (Kirk *et al.*, 1968; Ishihara & Miyazaki, 1972; Konishi *et al.*, 1974; Ander & Eriksson, 1976; Leonowicz *et al.* 1985; Kawai *et al.*, 1986; Szklarz · Leonowicz, 1986). For example in Eriksson 's laboratory, it was shown that the phenol oxidase-less (laccase-less) mutant of *Sporotrichum pulverulentum* obtained by UV-mutagenesis did not transform lignin while the wild type decomposed this polymer effectively(Ander & Eriksson, 1976). Also in our laboratory we have shown that *Trametes versicolor* laccase depolymerizes as well polymerizes lignosulphonates which constitute an adequate model of native lignin

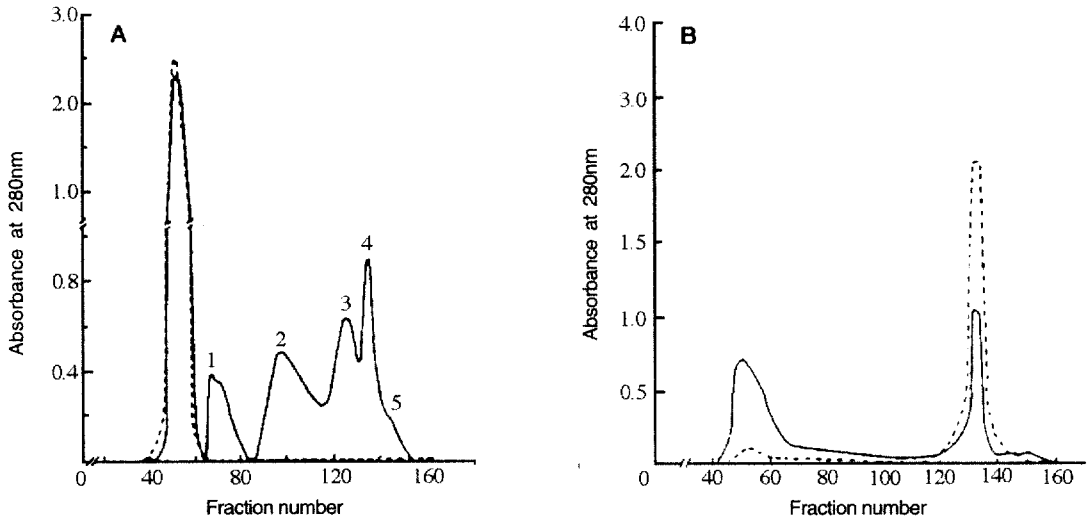

Fig. 4. Depolymerization (A) and polymerization (B) of Na-lignosuphonate fraction of the approximate molecular weights 97,000 (A) and 1,000 (B) daltons. Continuous line-elution profile on Sephadex G-50 column. Dashed line-elution profile of the control(Leonowicz *et al.*, 1985).

(Leonowicz *et al.*, 1985; see also Fig. 4).

The similar results with using lignosulphonates as the hydrogen donor for laccase isolated from fungus *Rigidosporus lignosus* were further found, however in the case of ^{14}C labelled Klason lignin the solubilization of the preparation by enzyme was insignificant, probably due to recondensation process(Galliano *et al.*, 1991).

3.2 Protocatechuate 3,4-dioxygenase (PCD)

The dioxygenase, widely distributed among bacteria, have also been identified in lignin decomposing fungi. Protocatechuate 3, 4-dioxygenase activity discovered in *Polystictus versicolor* by Haider *et al.* (1962) can serve as an example. In order to examine steps of the process initiated by dioxygenase catalyzing reaction in detail, radioactive vanillic acid was used.

The scientists from Kirk 's group (Chen *et al.*, 1983) formulated a thesis that among the many reactions occurring during the decay of lignin by white-rot fungi dioxygenase catalyzing reactions within the intact polymer take place. As a result, the peripheral aromatic ring of the

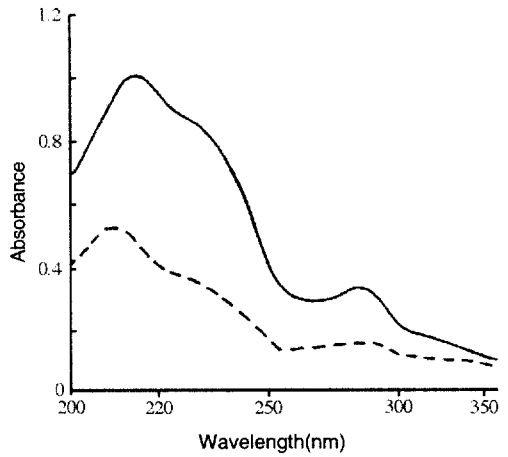

Fig. 5. The UV spectrum of Na-lignosulphonates (mol. weight approx. 97,000 daltons) before incubation with protocatechuate 3,4-dioxygenase (continued line) and after 3-h incubation with immobilized form of the enzyme (dashed line) (Wasilewska *et al.*, 1987).

polymer are opened and products of aliphatic carboxylic acid type are formed.

Protocatechuate 3,4-dioxygenase was further isolated from *Chaetomium piluliferum* and *Pleurotus*

ostreatus, and highly purified in our laboratory (Wasilewska & Trojanowski, 1980; Wasilewska *et al.*, 1987). The effect of this enzyme (and its immobilized form as well) on sodium sulphonates was examined(Wasilewska *et al.*, 1987). As a result a product was obtained which showed a significant lowering of the absorption spectrum in the region of 280 nm specific for aromatic rings. This indicated that dearomatization of the substrate took place(see Fig. 5).

3.3 Lignin peroxidase (LIP)

The lignin peroxidase (LIP, LPO) was discovered after many years of research in the culture of *Phanerochaete chrysosporium*. This enzyme was identified and described independently by Kirk's (Tien & Kirk, 1983), Gold's(Glen *et al.*, 1983) and Higuchi's(Shimada *et al.*, 1983) groups in low nitrogen, C-limited and flushed by oxygen growing medium of *Phanerochaete chrysosporium*. Lignin peroxidase(molecular weight around 42,000 daltons) requires hydrogen peroxide to be active. It catalyses several oxidations in the alkyl side chains of lignin-related compounds, C-C cleavage in side chains of lignin subunits, oxidation of benzyl alcohols to aldehydes or ketones, intradiol cleavage of phenylglycol structures and hydroxylation of benzylic methylene groups (Tien & Kirk, 1984). The enzyme is also able to cleavage aromatic ring via one-electron oxidation in lignin model compounds (Umezawa & Higuchi, 1987). Optimal activity of LIP occurs below pH 3. Firstly Tien and Kirk(1983) reported that the enzyme was specific only toward highly methylated lignin preparations obtained naturally (e.g. lignin separated from birch tree) and additively methylated by methyl iodide labelled in carbon (see Fig. 6).

Ten years later Tien's group using ^{14}C la-

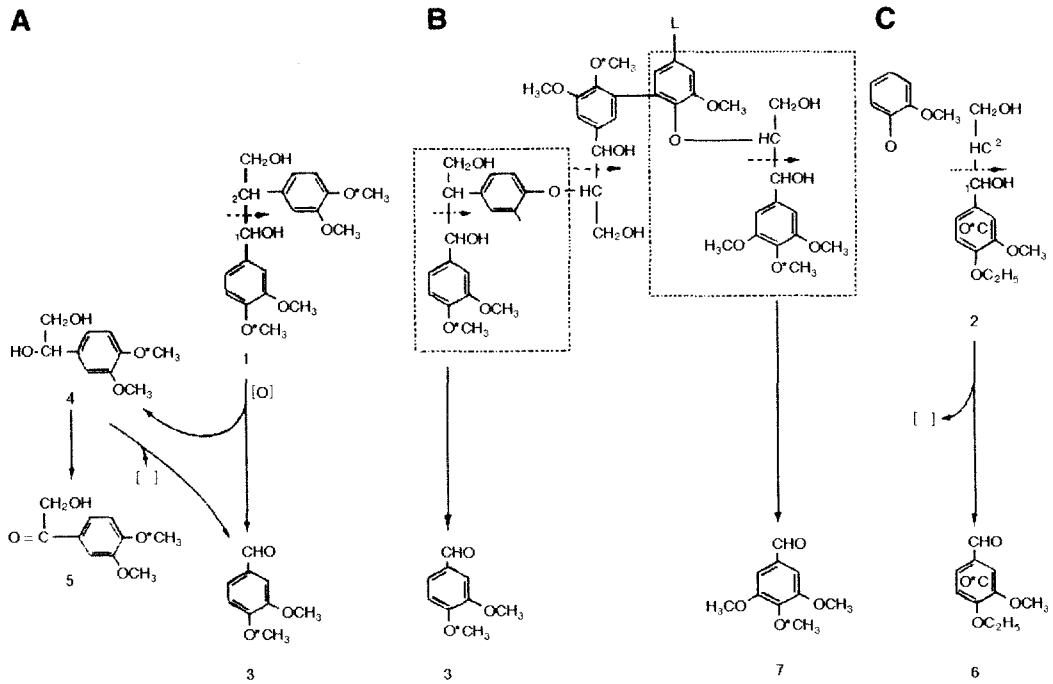

Fig. 6. LIP activity on a lignin preparation with artificially ^{14}C methylated phenolic hydroxyl groups. Dashed arrows signify the place where the links between subunits were broken (Tien & Kirk, 1983).

belled substrates unequivocally demonstrated that purified lignin peroxidase directly oxidizes and degrades synthetic lignin giving soluble lower mol. weight products (Hammel *et al.*, 1993). Against substrates containing an excess of free phenolic hydroxyl groups, the enzyme shows oxidizing and polymerizing activity as in the case of peroxidase. Veratryl alcohol, the substrate commonly used to assay LIP activity, must be extra-purified even from trace amount of phenolic compounds. Therefore, ligninase resembles "traditional" peroxidase(a similar molecular weight, hematin character of protein and requirement of hydrogen peroxidase for activity) but has extra-depolymerizing activity at low pH values.

3.4 Horseradish-like peroxidase (HRP)

Classical extracellular peroxidase (similar to horseradish peroxidase - HRP) by the first time was identified in growing culture of wood rotting fungus *Pholiota mutabilis* by Leonowicz and Trojanowski (1965). It (together with laccase) caused demethylation of vanillic acid and Björkman 's lignin. The enzyme was purified further from the culture filtrates of the white rot fungi, *Inonotus radiatus* and *Trametes versicolor* by Lobarzewski (1981). The enzyme constitution (molecular weight, hematin structure of the activity centre, complexing manner of hydrogen peroxide as a substrate) and many properties were similar to that of horseradish peroxidase. It did not show any activity on veratryl alcohol. The enzyme separated from *Trametes versicolor*, apart from apparent polymerization effect, depolymerized, though not to a large extent, the low-molecular fraction of Na-lignosulphonates at pH 3(see Fig. 7 B).

3.5 Manganese-dependent peroxidase (MnP)

Kuwahara *et al.* (1984) described a peroxidase whose activity was conditioned by the presence of manganese ions and named it manganese dependent peroxidase(MnP). Closer investigations carried out by Paszczynski *et al.* (1985) in Crawford 's laboratory showed that the enzyme cooperated with atmospheric oxygen under certain conditions. It does not only require hydrogen peroxide to act, but produces it in the process of glutathione, dithiothreitol or NADPH

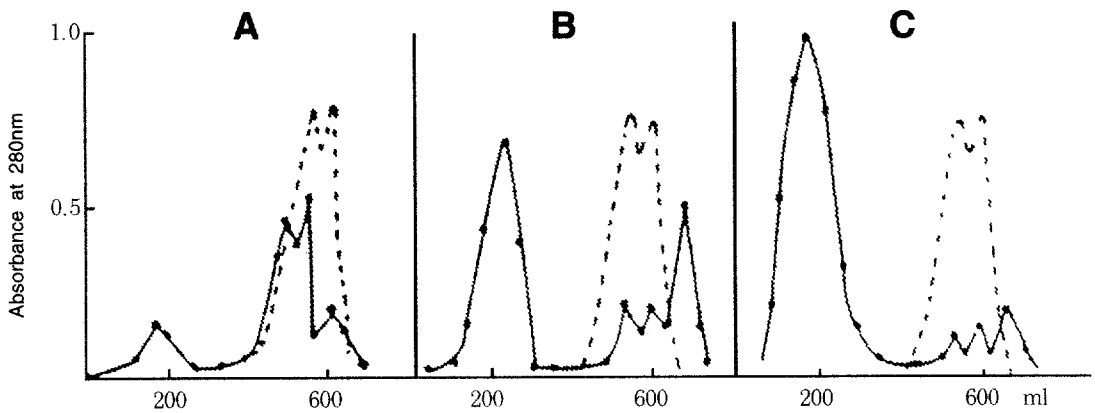

Fig. 7. The effect of immobilized *Trametes versicolor* peroxidase on Na-lignosulphonates. Continuous line indicates the elution profile of lignosulphonates after the reaction with peroxidase (A, B and C refer to various incubation periods; A:less than 12hr, B: 12 hrs, C: above 13hr). Broken line indicates the elution profile of the control Radicals and quinones generated by peroxidase (as in the case of laccase) may easily undergo polymerization and thus the depolymerization effect is less clear (Lobarzewski *et al.*, 1982).

oxidation. The molecular weight of the enzyme, 42~47 kilodaltons, is slightly higher than that of LIP I described by Kirk 's group. In the presence of hydrogen peroxide, the enzyme oxidizes hydrogen donors, typical for HRP, e.g. syringaldazine, guaiacol and syringic acid. However, it does not oxidize veratryl alcohol which differentiates it from typical lignin peroxidase. On the other hand, the enzyme demethylates some methoxyl compounds, e.g. 2-methoxy-3-phenylbenzoic acid, manifesting the activity crucial in lignin transformation.

3.6 Manganese-independent peroxidase (MIP)

This type of peroxidase was discovered by De Jong and coworkers (1992) in nutrient nitrogen-rich glucose/yeast extract/peptone medium of white rot fungus *Bjerkandera* sp.. According to Mester *et al.* (1996) MIP is inhibited by excess of manganese ions. The enzyme does not oxidize veratryl alcohol at all and can be determined by using 2, 6-dimethoxy-phenol as a hydrogen donor. Its substrate is hydrogen peroxide and centrum of activity contains an iron protoporphyrin prosthetic group(Kotterman *et al.*, 1994). Probably this type of peroxidase is similar to HRP-like one described by Lobarzewski(1981). However, up to date, no body studied its degradative ability on lignin, the enzyme, similarly as all other known peroxidases possesses probably such activity.

4. WHICH ENZYMES CAN COOPERATE WITH LIGNINOLYTIC ONES IN ATTACK ON THE LIGNIN BARRIER?

4.1 Glucose Oxidase(GOD)

Glucose oxidase (GOD) is the FAD dependent oxidase. In the enzymatic process, glucose is originally oxidized to gluconolactone with the simultaneous reduction of FAD to $FADH_2$. Then in the second stage, $FADH_2$ is oxidized to FAD by atmospheric oxygen and hydrogen peroxide is formed (Alberti & Klibanov, 1982).

During our screening studies it was reported that glucose oxidase occurs in majority of white rot fungi producing laccase and showing degradative ability on lignin. Almost all fungi deprived of such ability do not produce GOD at all (Leonowicz *et al.* 1986). Ramasamy and coworkers(1985) from a Reddy 's group reported that glucose oxidase negative mutants of *Phanerochaete chrysosporium* exhibit little or no ability to degrade lignin in comparison with the wild strain of fungus. On the other hand, also from the Reddy 's laboratory gone recently the report demonstrating production of laccase by Phanerochaete chrysosporium. In further part of this review one can read that glucose oxidase effectively cooperates with laccase in lignin degradation, so the results of Reddy 's group may point on GOD as an agent playing an important role in lignin degradation (Srinivasan *et al.*, 1995).

4.2 Cellobiose : Quinone Oxidoreductase(CBQ)

This FAD dependent oxidase (FAD as the prosthetic group) was identified in the culture filtrate of *Polyporus versicolor* and *Sporotrichum lignorum* (*Sporotrichum pulverulentum*) growing on cellulose as the sole carbon source by Westermark and Eriksson(1974), and highly purified from *Polyporus versicolor* culture filtrate(Westermark & Eriksson, 1975). CBQ reduces the qui-nones produced by laccase to respective phenols and oxidizes cellobiose generated by the cellulase complex to cellobionolactone(Westermark & Eriksson (1975).

4.3 Aryl alcohol oxidase(AAO) and particularly veratryl alcohol oxidase(VAO)

AAO is the FAD dependent extracellular oxidase. It was identified in the several cultures of ligninolytic white-rot fungi, mainly of *Trametes* and *Pleurotus* genera(de Jong *et al.*, 1994). The enzyme oxidize aromatic alcohols to aldehydes and reduces oxygen to hydrogen peroxide(see Fig. 8).

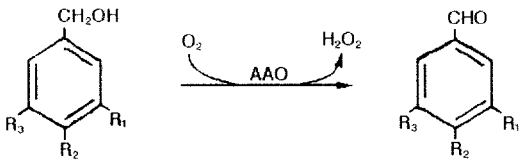

Fig. 8. Mechanism of oxidation of aromatic alcohols by AAO and VAO. R_1 - R_3 various groups characterizing particular alcohols. In the case of veratryl alcohol R_1=R_2=OCH_3, R_3=H (de Jong *et al.*, 1994).

AAO of *Pleurotus eringii* is a glycoprotein of a 72.6kDa and contains FAD as a prosthetic group. VAO have been found in the growing culture of *Pleurotus ostreatus* and characterized in Sannia 's laboratory by Sannia *et al.* (1991) and Palmieri *et al.* (1993). The enzyme is more specific for veratryl alcohol than other oxidases of AAO group.

4.4 Glyoxal oxidase(GLO)

The FAD-dependent glyoxal oxidase was discovered by Kersten and Kirk(1987) in the growing culture of *Phanerochaete chrysosporium* and characterized as the enzyme which can be activated by lignin peroxidase by Kersten *et al.* (1990).

4.5 Pyranose 2-oxidase(P2O)

FAD containing as a prosthetic group pyranose 2-oxidase was found in growing culture of *Phanerochaete chrysosporium*. The ultrastructural and immunocytochemical studies on the enzyme were done by Daniel *et al.* (1992).

4.6 1,4 benzoquinone reductase(BQR)

This FMN (Flavine mononucleotide) containing enzyme, found in *Phanerochaete chrysosporium* by Brock and Gold(1996), catalyzes the reduction of quinones to hydroquinones. The native enzymes was reduced quantitatively by NADH and the resulting reduced enzymes was reoxidized in the presence of one equivalent of 2,6-dimethoxy-1,4-benzoquinone(DMBQ). The enzyme probably may cooperate with laccase by a ping-pong steady-state mechanism.

5. HOW DO ENZYMES CAN COOPERATE IN DIRECT ATTACK ON THE LIGNIN MACROMOLECULE ?

Judging from what is already known about the very complicated structure of the lignin macromolecule, it is hard to image that this biopolymer can be effectively degraded by one enzyme only, even by laccase or lignin peroxidase. It seems only logical to accept a hypothesis suggesting a cooperation of some individual enzymes in the process of lignin degradation. It is well known, for instance, that cellulose, a biopolymer of a relatively simple structure (compared to lignin), requires for transformation a number of enzymes acting consecutively as the process of hydrolysis continues. The case of the lignin polymer may be quite similar. We have, in fact, had a chance to get to know some elements of such cooperation of active enzymes in reference to this polymer. As the lignin macromolecule is very big and complicated, the researchers very seldom worked with natural lignin preparations, and used rather the low molecular methoxyphenolic dimers or oligomers modeling some parts of lignin structure. After discovery of lignin peroxidase researchers looked after its direct activity on lignin macromolecule, but, when time passed it was more and more clear, that to reproduce lignin depolymerization by only one enzyme is a limited approach. From the other hand as it was known at this time that LIP needs hydrogen peroxide to be activated, people searched for enzymes providing this compound. Interestingly, one of the enzymes that generates hydrogen peroxide is an extracellular manganese dependent peroxidase. In this reason Paszczynski *et al.* (1985) considered the possibility of cooperation of both en-zymes helping each other in transformation of lignin

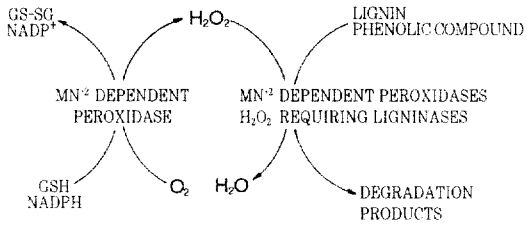

Fig. 9. Cooperation of MnP with LIP in ligninolysis (Paszczynski *et al.*, 1985).

related compounds (see Fig. 9).

A five years later Kersten *et al.* (1990) in Kirk's laboratory involving veratryl alcohol as the mediator of ligninolysis stated the possibility of cooperation of lignin peroxidase with to other two enzymes, namely glyoxal oxidase and manganese dependent peroxidase. Such a system may work in the culture of *Phanerochaete chrysosporium*. In this reason the lignin transformation to a lower molecular weight(LMW) pro-ducts goes throughout cation radicals found in their laboratory(see Fig. 10).

Also glucose oxidase as hydrogen peroxide donor can cooperate with lignin peroxidase. Such cooperation in decomposition of aromatic dimers by the system of two enzymes was shown in Gold's laboratory (Glen *et al.*, 1983). Glucose oxidase cooperates also with laccase. As we told already laccase alone can directly depolymerize lignin macromolecule, but the effectiveness of depolymerization depends on rapid removal of reactive radicals or quinones from the media that are formed by laccase (such phenoxy radicals, as we already told, are created, e.g. when laccase acts on 2,4,6-trimethoxyphenol). Spontaneous coupling of the radicals which are probably formed from phenolic fragments of lignin structure leads to the products of higher molecular weight(see Fig. 2). It has

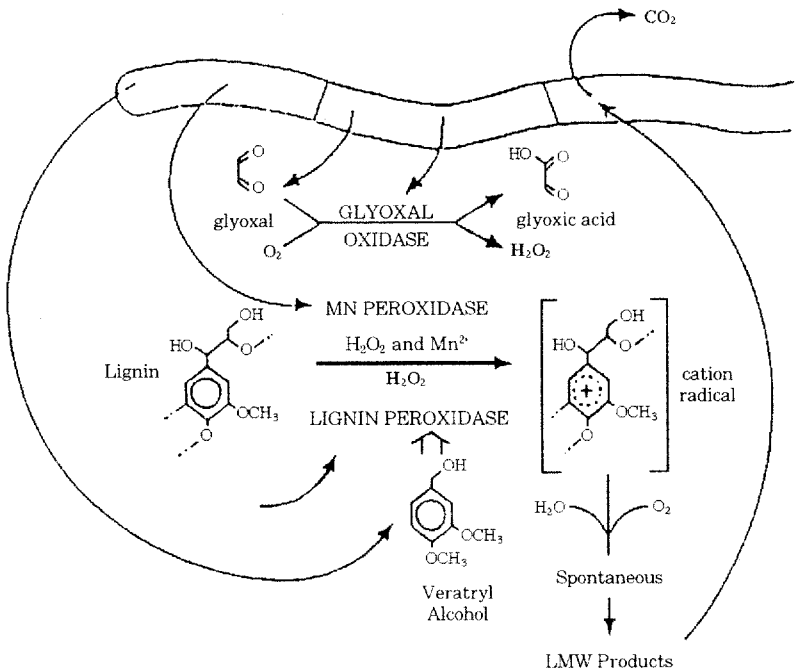

Fig. 10. The possible cooperation of three enzymes(GLO, MnP and LIP) in the ligninolysis by the culture of *Phanerochaete chrysosporium*(Kersten *et al.*, 1990).

turned out that glucose oxidase is a efficient factor which eliminates radicals and quinones from the laccase reaction medium by their reduction. It may function according to mechanism proposed by Green(1977).

The oxygen donors in glucose oxidation are quinones or radicals which are formed from phenolic compounds by laccase but not molecular oxygen. A similar course of reaction was also noticed by Alberti and Klibanov(1982) with regard to p-benzoquinone. The quinoid compound was reduced to p-hydroquinone by glucose oxidase.

We have investigated various phenolic compounds to examine their changes under the sequential activity of laccase and glucose oxidase(Leonowicz *et al.*, 1997). In all cases quinone products formed by laccase disappeared from the reaction medium when glucose oxidase was introduced into the reaction mixture. Oxidation of caffeic acid and reduction of the corresponding quinone can serve as an example (see Fig. 11).

Just recently Marzullo *et al.* (1995) in Sannia 's laboratory reported. that not only glucose oxidase but also other FAD dependent oxidase like veratric acid oxidase (VAO) can cooperate with laccase in transformation of some phenol-quinone derivatives. The enzyme as we told already is excreted by the white rot fungus *Pleurotus ostreatus*. The authors found that VAO is able to reduce laccase-generated quinoids, and phenoxy radicals with concominant oxidation of veratryl alcohol to veratryl aldehyde. It works also with synthetic quinones, e.g. with 2,6-dichlorophenoloindophenol(DCIP; see Fig. 12). In this reason DCIP was used both for assay of the quinone reduction and test of the effect of oxygen and veratryl alcohol on VAO Activity.

These results show a certain regularity which may, in fact, be applicable to the lignin trans-

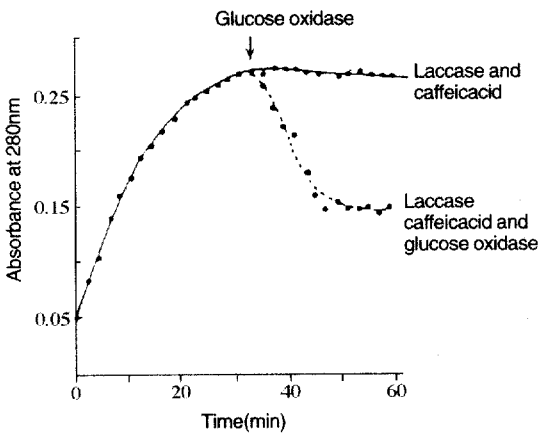

Fig. 11. Formation and reduction of quinone under subsequent laccase and glucose oxidase effects on caffeic acid (Leonowicz *et al.*, 1997b).

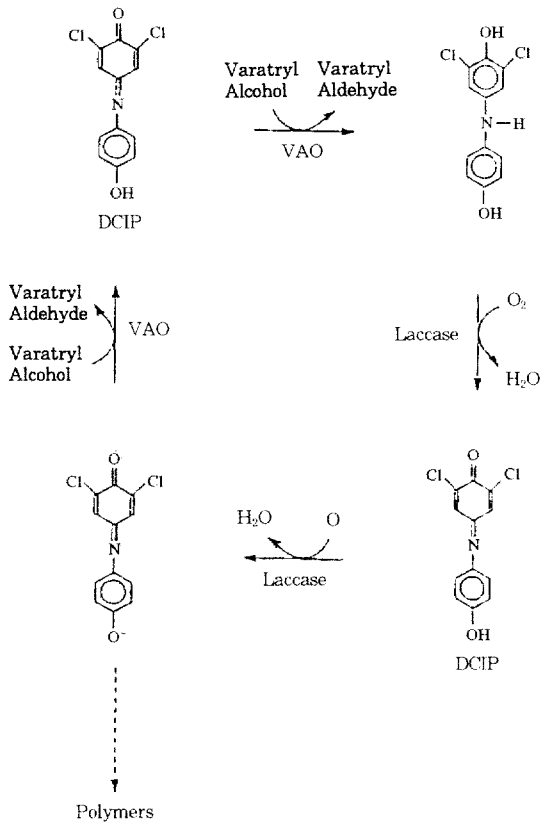

Fig. 12. The possible VAO-laccase cycle, when DCIP serves as the substrate of both enzymes(Marzullo *et al.*, 1995).

formation. The first such attempts using laccase and glucose oxidase was made in our laboratory. Two fractions of lignosulphonates(Peritan Na) which constitute an adequate model of native lignin were applied (Szklarz & Leonowicz, 1986). It turned out that GOD added to the reaction mixture containing lignosulphonates and laccase, effectively counteracted polymerization of these substrates on the one hand and accelerated depolymerization processes catalyzed by laccase on the other(see Fig. 13).

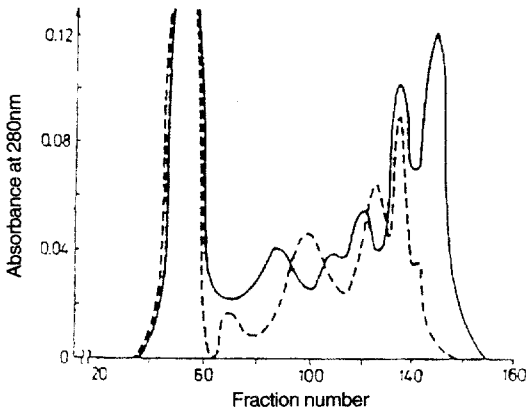

Fig. 13. Elution pattern of the high-molecular fraction of Peritan Na on a Sephadex G-200 "superfine" column after incubation with both LAC and GOD. The control (omitting glucose and glucose oxidase) is indicated by a dashed line (Szklarz & Leonowicz, 1986).

Such an effect of laccase and glucose oxidase working in concert in the process of lignin degradation was further fully confirmed by Galliano *et al.* (1991). The authors used their own [^{14}C] labelled Klason lignin isolated from [^{14}C] labelled ligninocellulose. The last biopolymer was taken from young Hevea twigs grown in the medium provided with [^{14}C] phenylalanine.

Instead of glucose oxidase veratryl alcohol oxidase may cooperate with laccase (Marzullo *et al.*, 1995). Its cooperative action simillarly as in the case of glucose oxidase prevented the polymerization of phenolic compounds and reduced the molecular weight of soluble lignosulphonates to a significant extent.

Also manganese dependent peroxidase can cooperate with laccase, what was unequivocally stated in the report of Galliano *et al.* (1991). When MnP and LAC isolated from the culture of *Rigidosporus lignosus* were added to the medium containing radioactive Klason lignin at the same time, lignin degradation was intensive. The addition of glucose oxidase to such enzyme system significantly increased the quantity of low molecular products. In the presence either LAC or MnP alone, lignin solubilization was relatively limited. This was the first demonstration of the synergic effects of two oxidizing enzymes involved in lignin biodegradation.

6. HOW DO ENZYMES BE ABLE TO COOPERATE IN ATTACK ON THE LIGNINOCELLULOSE COMPLEX ?

It has been unequivocally stated that lignin degradation is accelerated in the presence of cellulose or its oligomers (Hatakka & Uusi-Rauva, 1983). The idea of interdependence of delignification and cellulose degradation processes was postulated for the first time by Westermark and Eriksson (1974). This hypothesis is still valid as the reports by Gottlieb *et al.* (1950) concerning the possibility of mycelial growth on lignin as the sole source of carbon have not confirmed. According to Westermark and Eriksson depolymerization processes of cellulose and lignin are interrelated in certain points and accelerate each other(Westermark & Eriksson, 1974a · 1974b · 1975). They discovered the enzyme CBQ which cooperates with laccase and cellulose in the process of depolymerization of both components of the lignocellulose complex in a feedback fashion. CBQ, accelerating degradation of both elements of the complex in a feedback system, thus removes decomposition products of cellulose. The authors suggested that in the sys-

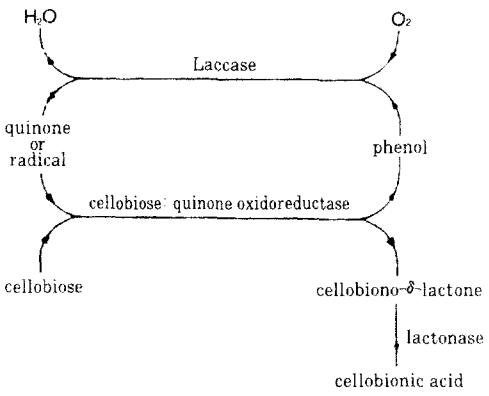

Fig. 14. A proposed mechanism for cellobiose : quinone oxidoreductase(Westermark & Eriksson, 1974b).

tem laccase might function as a link in an extracellular "electron transport chain" (see Fig. 14). On the other hand, we showed that the products of partial wood hydrolysis distinctly induced enzymatic system which degrade both constituents of the ligninocellulose complex (Szczodrak *et al.*, 1986).

It is necessary to mention that the lignin peroxidase regarded crucial in the degradation of lignin was not known when CBQ was discovered. It is well known that LIP requires hydrogen peroxide to act, the compound which is not produced in the CBQ system. Therefore it seems that one should look for a natural system generating this factor. And this is precisely what we looked for in our laboratory.

Various possibilities were considered. First of all, the function of some cytosolic fungal enzymes was analyzed, e.g. glyoxal oxidase, veratryl alcohol oxidase, manganese-dependent peroxidase or glucose oxidase. These enzymes can function both in the cytoplasm and extracellularly. Other enzymes generating hydrogen peroxide like pyranose-2-oxidase, methanol oxidase, and fatty aryl-CoA oxidase as only intracellular(de Jong *et al.*, 1994) can not be considered, because wood transformation processes go extracellularly. The firstly listed enzymes are relatively stable and resistant to outside factors. They are known as a rather defense in lignolysis but cooperating with other enzymes in transformation of lignin. But among all these enzymes only glucose oxidase can join both lignin and carbohydrate metabolic chains of ligninocellulose complex. It means GOD possesses two necessary functions for acceleration of ligninocellulose metabolism, namely by oxidation of glucose produces hydrogen peroxide necessary for LIP activity, and reduces quinoids and phenoxy radicals yielded by laccase during oxidation of lignin. Other listed above enzymes although may reduce quinones and produce hydrogen peroxide can not be taken into consideration, because hydrogen peroxide production does not go from oxidation of glucose, which is a key metabolite in transformation of carbohydrates. It means that only GOD cooperates with the system of cellulases oxidizing glucose, generated by these enzymes during cellulose hydrolysis(Leonowicz *et al.*, 1997b). The enzyme is produced by all white-rot fungi yielding laccase and showing ability on lignin degradation(Leonowicz *et al.*, 1986). Also it was found in many known up to date fungi producing lignin peroxidase and in *Phanerochaete chrysosporium* too (Kelley & Reddy, 1988). This fungus, the best lignin transformer, deprived of glucose oxidase by mutagenization does not decompose lignin at all (Ramasany *et al.*, 1985). On the other hand LIP produced by this fungus directly destroys lignin macromolecule (Hammel *et al.*, 1993). *Phanerochaete chrysosporium* together with lignin peroxidase possesses also laccase what was recently unequivocally stated by Reddy 's group (Srinivasan *et al.*, 1995).

The oxygen indispensable in the second stage of glucose oxidase-glucose reaction(see Fig. 16) can be supplied by radicals or quinones generated when phenolic or methoxyphenolic compounds are exposed to laccase activity (Szklarz & Leonowicz, 1986). It was also shown that quinones produced by laccase are inhibitors of

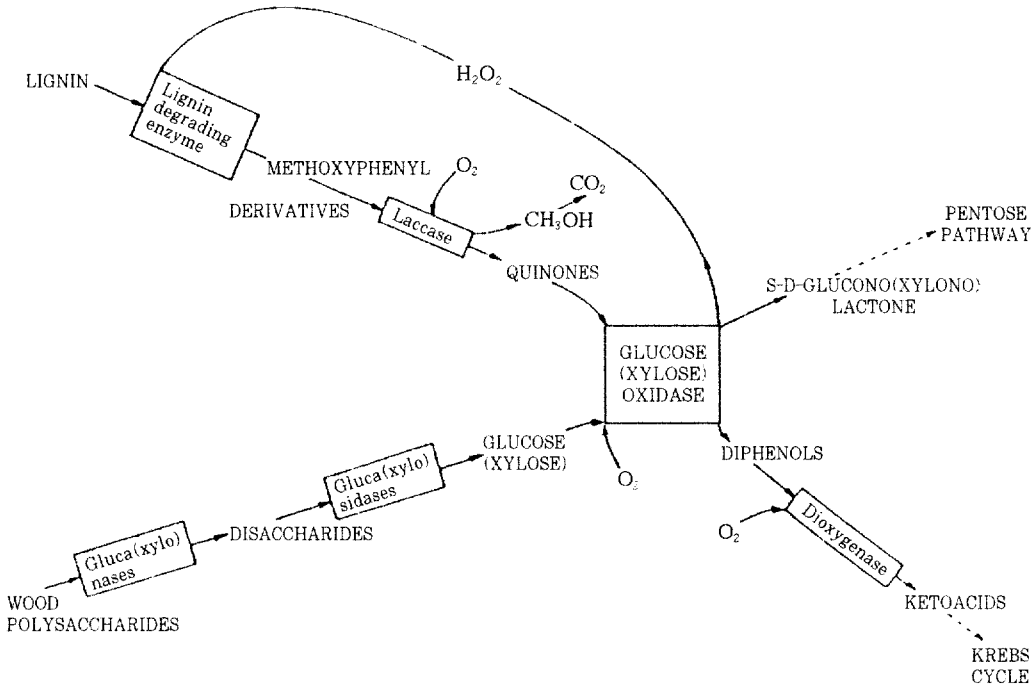

Fig. 15. The hypothetical mechanism of ligninocellulose transformation by white-rot fungi enzymes (Leonowicz *et al.*, 1986).

the enzyme(Szklarz & Leonowicz. 1986). Therefore, it can be concluded that glucose oxidase counteract the poisonous quinones in the medium, enabling laccase to continue its function.

7. CONCLUSION

Considering our results and those of other authors we can conclude that the general outline for the complementary character of carbohydrates and lignin decomposition and the existence of enzymatic systems combining these processes is still topical although it was formulated and propounded by Eriksson 's group over than 20 years ago, at the time when lignin peroxidases have not yet been discovered. So far, our postulated eleven years ago hypothetical mechanism of transformation of ligninocellulose complex (Leonowicz *et al.*, 1986) is still actual. In this reason let us show it in Fig. 15 with slight necessary modifications in comparison to the original presented on Pulp and Paper Conference in Stockholm (Leonowicz *et al.*, 1986).

Let us remain the more important theses and routes of this mechanism:

- GOD cooperates with LIP providing hydrogen peroxide and with LAC reducing yielded by this enzyme quinones to adequate phenols, so, GOD capable of generating hydrogen peroxide operates as a feedback system where LIP functions as the first lignin decomposing agent and laccase as the demethylating factor;
- Glucose which is produced as a result of cellulose hydrolysis by the cellulase complex becomes the substrate for GOD;
- Oxygen which is indispensable to carry out the reaction can come from quinones produced by LAC from lignin oligomers;

- Gluconolactone formed as a result of glucose oxidation, after certain transformations, reinforces the metabolism of the fungus in the pentose phosphate cycle or in glycolysis;
- Hydrogen peroxide produced in the reaction catalyzed by GOD, in turn, activates LIP ;
- Lignin exposed to LIP undergoes decomposition into fragments containing methoxyl groups;
- LAC demethylates oligomers yielded by LIP and degrade them to even lower fragments;
- Generation of the excess amount of quinones and possible secondary polymerization is counterbalanced by GOD which reduces these to respective phenols;
- The phenols, in turn, become the substrate for dioxygenase(PCD), present in fungal cultures, which calalyse cleavage reactions of the aromatic rings, the products obtained in the form of ketoacids easily find their way to the Krebs cycle.

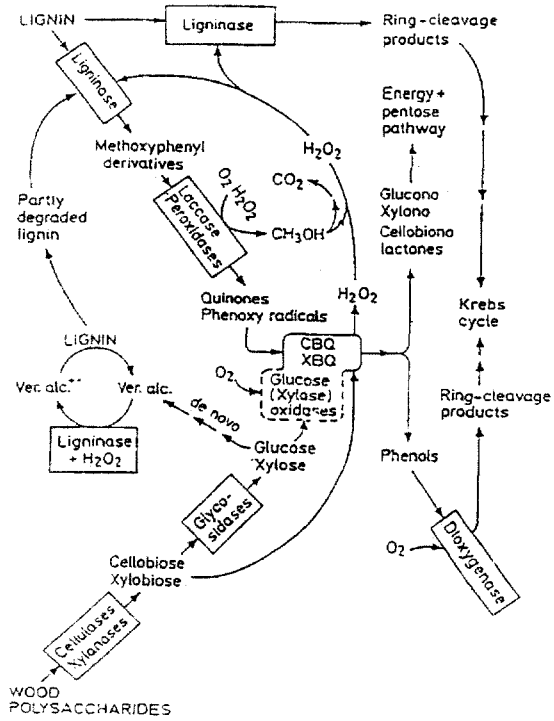

Fig. 16. Hypothetical scheme for degradation of lignin, cellulose and xylan in wood (Eriksson *et al.*, 1990).

The above mechanism could occur in those fungi which possess all the mentioned enzymes, e.g. *Phanerochaete chrysosporium*, *Phlebia radiata*, and *Trametes versicolor*. For those species in which the presence of LIP has not been confirmed although they transform ligninocellulose, the mechanism suggested by Westermark and Eriksson seems to function very well. According to them CBQ is the crucial enzyme in the process. It is not out of the question that both systems function in concert as was shown in Eriksson 's laboratory and published in a monograph on wood microbial transformation(Eriksson *et al.*, 1990) and in a mini-review article (Eriksson, 1993;see Fig. 16).

As it can be seen our scheme was developed by Eriksson 's group by adding CBQ activity and other details. It firmly enriched its areas of function(not one, but two enzymes of feedback activity). We are in agreement with this proposition. However, the existence of other, still to be discovered, mechanisms as well as other enzymes function as feedback systems in the processes of ligninocellulose transformation is not ruled out. Close investigations of these processes would definitely help to solve the problem of how to utilize the supply of waste lignocellulose which is accumulated as a result of man 's activity. This, however, seems to be the problem left for the generations to come.

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