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Decay mechanisms of brown-rot fungi

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ABSTRACT

Brown-rot fungi, e.g. the dryrot fungus (*Serpula lacrymans*), are the most harmful microorganisms in wood in service in Finland and in temperate regions. Brown-rot fungi cause wood decay primarly by attacking the carbohydrates of the cell walls, leaving lignin essentially undigested.

At the initial stage of the decay, the brown-rot fungi seem to operate by a mechanism which cause extensive changes in the wood cell wall structure, leading to a rapid decline in the strength properties. It has been suggested that brown-rot produce a low molecular degradation agent which is capable of penetrating into the cell wall structures. Research on the brown-rot decay mechanism has focused on identifying the low molecular weight compounds enchancing cellulose depolymerization in the initial stages of brown-rot decay. The production of extracellular hydrogen peroxide by brown-rot fungi was qualitatively and quantitatively detected by using chromogen ABTS (2,2-azinobis(3-ethylbenzthiazoline-6-sulphonic acid)) and horseradish peroxidase. Two brown-rot fungi, *Poria placenta* and *Serpula lacrymans* were found to produce hydrogen peroxide on solid spruce sawdust medium. The production of hydrogen peroxide by *P. placenta* was observed in liquid culture media containing either amorphous or crystalline cellulose as a carbon source. The production of hydrogen peroxide and oxalic acid occured to be simultaneous on crystalline and amorphous cellulose, and the highest amount of hydrogen peroxide was detected on amorphous cellulose. The production of hydrogen peroxide by *P. placenta* dependent on the formation of acid pH of the culture medium. The accumulation of hydrogen peroxide was preceeded by a drop of pH of the culture medium, which was due to the production of oxalic acid. As a small diffusible molecule, hydrogen peroxide can act as degradation agent providing reactive hydroxyl or other oxygen radicals through the Fenton type of reaction which leads to the degradation of wood cellulose.

The enzymatic hydrolysis of wood polysaccharides by *Gloeophyllum trabeum* was detected by following the production of cellulases, hemicellulases and extracellular protein on spruce sawdust or microcrystalline cellulose media. The production of endo-β-1,4-glucanase and endo-β-1,4-xylanase was most pronounced on both media. Brown-rot fungi differ from other cellulolytic fungi by lacking enzyme activites needed for the enzymatic degradation of crystalline cellulose. The endoglucanase activities produced by *P. placenta* were most

pronouced on glucose medium, thus indicating that the brown-rot cellulases are constitutive and not repressed by glucose. The degradation of hemicellulose is believed to be an important initial reaction taking place in the brown-rot decay. The endo-β-1,4-xylanase produced by *G. trabeum* was purified and characterized and appeared to be a protein with a molecular mass of 39 - 42 kDa. The endo-β-1,4-xylanase of *G. trabeum* has its pH optimum at pH 4 and it is found to have a very high temperature optimum (80^{o}C).

A biomimetic approach was used to clarify the role and importance of the Fenton-type reaction in the carbohydrate degradation by brown-rot fungi. Spruce sawdust and microcrystalline cellulose were modified in the H_2O_2/Fe(II) treatment. The degree of hydrolysis of the pretreated spruce sawdust was clearly increased with complete cellulase (Econase), purified endogluganase from *Trichoderma reesei* and endoglucanase of *P. placenta*. The oxidative pretreatment of microcrystalline cellulose decreased the hydrolyzability of pure cellulose with the complete cellulase, but the hydrolyzability with both purified endoglucanase *of T. reesei* and endoglucanase from *P. placenta* was increased. Thus, after oxidative treatment with Fenton´s reagent the hydrolysis of both pure cellulose and wood was substantially increased.

PREFACE

This study was carried out at VTT Building Technology, Wood Technology, and VTT Biotechnology and Food Research of the Technical Research Centre of Finland (VTT) during the years 1989 - 1995 as a part of two larger projects called " Natural Wood Preservation " and "Bio Decay". The former project was financed by Technology Development Centre (TEKES) and Finnish Wood Research Ltd. and the latter by Technology Development Centre (TEKES) and the consortium of Finnish Wood Companies, Osuuskunta Metsäliitto, Kymmene Oy and Koskisen Oy. Their financial support is gratefully acknowledged.

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APPENDICES

Appendices of this publication are not included in the PDF version. Please order the printed version to get the complete publication (http://www.inf.vtt.fi/pdf/publications/1996)

LIST OF PUBLICATIONS

This thesis is based on following publications (Appendices I - IV) which are referred to as I - IV in the text.

I Ritschkoff, A.-C. & Viikari, L. (1991): The production of extracellular hydrogen peroxide by brown-rot fungi. Material und Organismen 26: 157 - 167.

II Ritschkoff, A.-C., Buchert, J. & Viikari, L. (1992): Identification of carbohydrate degrading enzymes from the brown-rot fungus, *Gloeophyllum trabeum*. Material und Organismen 27: 19 - 29.

III Ritschkoff, A.-C., Buchert, J. & Viikari, L. (1994): Purification and characterization of a thermophilic xylanase from the brown-rot fungus *Gloeophyllum trabeum*. J. Biotechnol. 32: 67 - 74.

IV Ritschkoff, A.-C., Rättö, M., Buchert, J. & Viikari, L. (1995): Effect of carbon source on the production of oxalic acid and hydrogen peroxide by brown-rot fungus *Poria placenta*. J. Biotechnol. 40: 179 - 186.

ABBREVIATIONS

ABTS	2,2-Azinobis(3-ethylbenzthiazoline-6-sulphonic acid)
CBH	Cellobiose hydrolase
CDH	Cellobiose dehydrogenase
CMC	Carboxymethyl cellulose
EG	Endoglucanase
ELISA	Enzyme-linked immunosorbent assay
ESR	Electron spin resonance spectroscopy
FDH	Formate dehydrogenase
HEC	Hydroxyethyl cellulose
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
KTBA	2-keto-4-thiomethylbutyric acid
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide
NMR	Nuclear magnetic resonance spectroscopy
ODC	Oxalate decarboxylase
$OH^{\cdot}$	Hydroxyl radical
SEM	Scanning electron microscopy
TCA	Tricarboxylic acid cycle
TEM	Transmission electron microscopy

1 INTRODUCTION

As an organic and heterogenous material wood is very susceptible to damage caused by different microorganisms, such as decay fungi, mould and bluestain fungi and bacteria. Wood decay fungi can be divided into three different groups; brown-rot, white-rot and soft-rot fungi. Brown-rot fungi cause wood decay primarily by attacking the carbohydrates of the cell wall, whereas white-rot fungi can attack both the carbohydrates and lignin in the cell wall. Soft-rots are caused by microfungi that selectively attack the S_2 portion of the cell wall. Mould and bluestain fungi grown on the surface of moistened wood utilize available simple carbon compounds, but do not decline the strength properties of wood (Zabel & Morrell, 1992). The most severe damages are caused by decay fungi since they affect directly to the strenght properties of wood. Brown-rot fungi, e.g. *Serpula lacrymans* and *Coniophora puteana*, the most harmful microorganisms occurring in wood used in Finland and temperate regions in general, prefer softwoods to hardwoods as their substrates. Especially the dry rot fungus *S. lacrymans*, which is able to transport water efficiently trough mycelial strands from the source of moisture into dry wood, is a common, destructive brown-rot fungus reported to occur in wooden constructions in Europe (Campbell, 1952, Viitanen & Ritschkoff, 1991, Viitanen, 1994).

The chemical and physical structure of wood cell wall affects greatly to the type and intensity of decay (Jeffries, 1987). The natural durability of different wood species is very variable. In general, the Finnish wood species are relatively undurable. The heartwood of *Pinus sylvestris* resists microbial attack better than the sapwood (Viitanen & Ritschkoff, 1991). It has also been shown that wood components from a living tree are more durable than wood material in use. The natural durability of wood is affected by several factors, such as the permeability of moisture and the amount and composition of wood extractives (von Erdtman & Rennerfelt, 1944; Findlay, 1951, Rudman, 1963).

1.1 WOOD AS A NATURAL SUBSTRATE FOR DECAY FUNGI

1.1.1 Chemical and physical structure of wood

The wood cell wall consists of three main macromolecular components; cellulose, hemicellulose and lignin. In the wood cell wall there are also minor low-molecular weight components, extractives and mineral substances, which vary in different wood species in kind and amount. (Fengel & Wegener, 1989).

Cellulose, as the most important component of cell wall, constitutes approximately one half (40 %- 45 %) of both softwoods and hardwoods. Chemically simple but physically rather complicated homopolysaccharide cellulose is located mainly in the secondary cell wall. This wood polysaccharide consists of D-glucopyranose units linked by β-1,4-bonds. The linear cellulose molecules have a strong tendency to form intermolecular and intramolecular hydrogen bonds (Sjöström, 1981, Fengel & Wegener, 1989). The degree of polymerization (DP) determines the molecular size of cellulose and thus, the number of glucose units in one cellulose molecule. Their larger aggregates, microfibrils and fibrils, formed by glucose molecules are bound together with hydrogen bonds ensure the enormous strength properties of the structure (Fengel & Wegener, 1989).

Cellulose is composed of well ordered crystalline regions as well as of amorphous, less ordered parts. In the solid state the hydrogen bonds between cellulose molecules are not arranged irregularly or at random, but the regular system of the hydrogen bonds results in an ordered system with crystalline-like properties (Fig. 1) (Fengel & Wegener, 1989). The structure of crystalline cellulose is very durable against the microbial action (Eriksson et al., 1990, Jeffries, 1987, Sjöström, 1981). Cellulose can fulfil its function as the main structural component of the plant cell walls because of its chemical and physical properties (Fengel & Wegener, 1989).

Hemicelluloses are in close association with cellulose in the cell wall. The sturctures of the different types of hemicelluloses depend on the plant species. Hemicelluloses are named according to the main sugar residues in the backbone, e.g. xylans, glucomannans, galactans and glucans. The chemical structure of hemicellulose is quite complicated. It is composed of branched heteropolymeric substances, which are made up of glucose, xylose, galactose, mannose, arabinose and 4-0-methylglucuronic acids of glucose and galactose. The hemicellulosic substituents can be acetylated depending on the wood species (Sjöström 1981, Fengel & Wegener,1989, Viikari et al., 1993).

Galactoglucomannans form the main part of hemicellulose in softwoods. The backbone is composed of 1,4-β-linked D-glucose and D-mannose units, which are distributed randomly within the molecule. D-galactose side-groups are attached to mannose and glucose units via α-1,6-bonds. O-Acetyl-galactoglucomannans are the principal hemicelluloses in softwoods, and they can be divided into two main fractions based on galactose: glucose: mannose ratios. The fraction with low galactose content is also often referred to as glucomannans (Timell, 1967).

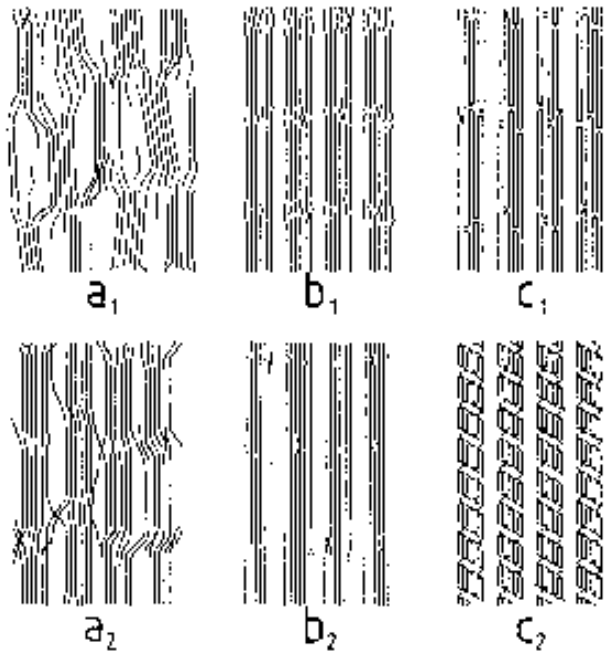

Fig. 1. The structural properties of cellulose fibrils (taken from Fengel & Wegener, 1989). Three basic models for the arrangement of cellulose molecules within the fibrillar units (a_1-c_1), and respective variations (a_2-c_2).

a) *Ordered regions with transitional molecular chains changing from one ordered region to another.*

b) *Individual fibrillar units with a sequence of ordered and less ordered regions.*

c) *Fibrillar units consisting of folded cellulose chains.*

Xylans, possessing a β-1,4-linked xylopyranosyl backbone, are present in all terrestrial plants and comprise up to 30 % of the cell wall material of annual plants, 15 - 30 % of hardwoods and 7 - 10 % of softwoods (Sjöström, 1981). Xylans are usually located in the secondary cell wall. The xylan in softwoods is mainly arabino-4-O-methyl glucuronoxylan, which in addition to 4-O-methyl glucuronic acid is also substituted by L-arabinofuranoside units linked by α-1,3-glycosidic bonds to the xylan backbone (Fig. 2). The ratio of arabinose side-groups to xylose side-groups in softwood xylan is 1:8. No acetyl groups are attached to softwood xylans. Arabinoxylan typical for softwood contains approximately 18 % of 4-0-methyl-D-glucuronic acids, whereas the xylan in hardwood is mainly 0-acetyl-4-0-methylglucuronic xylan. In most plant species the role of xylan is to act as intercellular substance separating cellulose and lignin (Timell, 1967, Sjöström 1981, Fengel & Wegener, 1989).

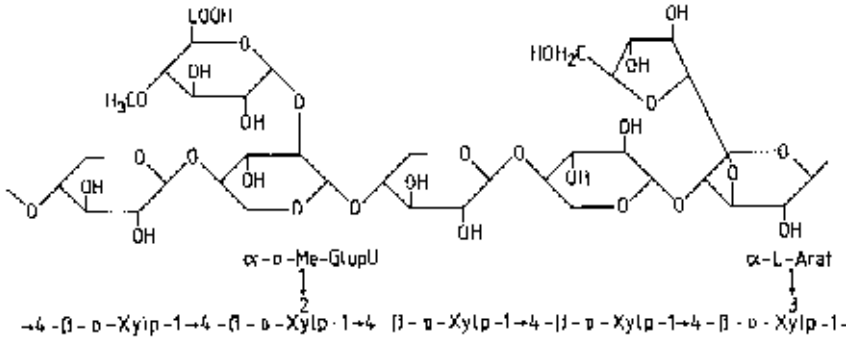

Fig. 2. Partial chemical structure of arabino-4-O-methylglucuronoxylan from softwood (taken from Fengel & Wegener, 1989).

The third structural component of wood cell wall, lignin, is a complex, aromatic and amorphous polymer made up of nonrepeating phenylpropane units linked by various carbon - carbon and ether bonds (Rypacek, 1977, Sjöström, 1981, Barr & Aust, 1994). The composition of lignin varies according to wood species. The lignin in softwoods consists of coniferyl alcohols and in hardwoods the lignin contains almost equal amounts of coniferyl and sinapyl alcohols. The main role of lignin is to act as glueing material in the cell wall and to give the plant sufficient rigidity (Sjöström, 1981).

1.1.2 Brown-rot fungi in wood

Brown-rot fungi utilize hemicellulose and cellulose in the cell wall, leaving lignin essentially undigested (Cowling, 1961, Micales & Highley, 1988). The brown-rot fungi have been observed to modify lignin, as indicated by demethylation and accumulation of oxidized polymeric lignin-degradation products (Jin et al., 1990a, 1990b). However, the recent ^{13}CPMAS NMR studies have been unable to produce evidence of demethoxylation or detectable loss of lignin in brown-rotted samples (Kim & Newman, 1995, Newman & Kim, 1995). The fungi cause wood darken, shrink, and break into brick-shaped pieces that crumble easily into a brown powder. The fungal hyphae penetrate from one cell to another through existing pores in wood cell walls early in the decay process. The penetration starts from the

cell lumen where the hyphae are in close connection with the S_3 layer (Highley, 1987a, Viitanen & Ritschkoff, 1991).

In brown rot, the decay process is thought to affect the S_2 layer of the wood cell wall first, while the S_3 layer remains intact until the late phases of decay. Furthermore, the porosity of the S_3 layer remains relatively unchanged during the decay process (Highley & Murmanis, 1985, Highley & Murmanis, 1987, Kuo et al., 1988). However, Green and Highley (1995) have recently questioned the results obtained in TEM and SEM studies concerning the intactness of the S_3 layer. The shape of the decayed wood cell remains constant due to the network formed of lignin residue (Highley et al., 1983a).

Chemical analysis of brown-rotted white pine (*Pinus monticola*) and hard maple (*Acer rubrum*) wood showed that the hemicellulose glucomannan is removed considerably faster than cellulose or xylan (Highley, 1987a). Xylan is usually depleted faster than cellulose. Since hemicelluloses form an encrusting envelope around the cellulose microfibrils, further degradation and removal of cellulose may depend on previous removal of the hemicelluloses. Thus, hemicellulose utilization may be a critical initial step during growth of brown-rot fungi in wood (Green & Highley, 1995). White-rot fungi successively depolymerize cell wall carbohydrates only to the extent that the products are used in fungal metabolism. These fungi produce a gradual decrease in the average degree of polymerization (DP) of holocellulose during all stages of decay but only slightly change the solubility properties of wood (Cowling, 1961, Green & Highley, 1995). Brown-rot fungi, in contrast to white-rot, rapidly depolymerize holocellulose, and the degradation products are formed faster than they are utilized (Cowling, 1961). Brown-rot fungi cause a more rapid drop in strenght properties than white-rot fungi, which reflects a higher degree of depolymerization of holocellulose in brown-rotted wood (Green & Highley, 1995).

1.2 GROWTH REQUIREMENTS OF BROWN-ROT FUNGI

1.2.1 Moisture and temperature conditions

One of the most important factors promoting fungal growth and development is a sufficient moisture content in woody materials. Most studies have demonstrated that the necessary humidity for the development of brown-rot fungi must be above 95 - 98 % for a long period and the wood moisture content around the fibre saturation point, 25 - 30 % water of the wood dry weight (Amner, 1964, Boddy, 1983, Cartwright & Findlay, 1946, Griffin, 1977, Viitanen, 1994). The formation of free water layer in the wood cell lumen provides essential gas conditions for the growth of fungal cell (Deacon, 1984). The moisture requirements, however, vary considerable depending on the fungal species. The optimum moisture

content for the growth of brown-rot fungi is 30 - 80 %. The moisture content of wood increases during the progress of decay, in some cases it can reach 150 - 250 % (Cartwright, 1929, Viitanen & Ritschkoff, 1991, Viitanen, 1994). The system by which brown-rot fungi regulate the moisture content of the substrate is not well-known. However, fungal water economics is affected by the fungal species, physiological status of the fungus and the properties of the wood material (Viitanen & Ritschkoff, 1991). According to some studies, most brown-rotters are fairly sensitive to moisture conditions, especially at the initial state of the decay, which could be due to the limited amount of oxygen in the wood material (Cartwright, 1929 , Hui-Seng & Eriksson, 1985).

The temperature needed for brown-rot fungal activity usually varies between 0 - 45 ^{o}C, depending on the fungal species, indicating the mesophilic characeristics of the most fungal species (Bech-Andersen, 1987, Harmsen, 1982, Viitanen, 1994). The growth of the decay fungi can initiate at + 0 ^{o}C and the fungal metabolic activity and growth rate increase with increasing temperature (Boddy, 1983, Griffin, 1981). High temperatures have been observed to act as restricting factor for fungal growth. However, the thermotolerance of fungi is determined by the developmental stage and age of the fungal colony, and thus high temperature is endured better by old mycelia than by young ones (Kubric & Wazny, 1978).

1.2.2 Nutritional requirements

The optimal nutritional needs of wood decay fungi vary, but the basic requirements are satisfied by the structural carbohydrates, certain inorganic compounds and vitamins in wood. In laboratory conditions the successful cultivation takes place on simple media, such as malt extract or potato dextrose, and artificial media containing essential inorganic substances together with a suitable carbon source. Successful colonization of wood depends largely on the ability of fungi to spread rapidly by using nonstructural carbohydrates (Deacon, 1984, Green & Highley, 1995).

Nitrogen is a critical factor in the growth of wood-decay fungi. The sparsity of nitrogen in wood, about 0.03 to 0.10 %, indicates that wood-decay fungi have an efficient mechanism for nitrogen metabolism and reuse (Merrill & Cowling 1966, Levi et al., 1968, Highley, 1987a). Micales (1992a) observed that the protease production by *Postia* (=Poria) *placenta* was most pronouced in the late growth phase of the mycelia, corresponding to autolysis, thus, indicating that the majority of protease activity is utilized for the reuse of fungal proteins rather than for the breakdown of extracellular nitrogenous substrates. Field evidence (Larsen et al., 1978) and results in vitro culture studies support the theory that wood-decay Basidiomycetes can conserve and function with the small amount of nitrogen in their own mycelia or by the lysis of other fungi in wood during decay, together with an extremely economical use of nitrogen in metabolism. However, other sources of nitrogen, such as obtained through bacterial fixation, may sometimes be required to form sporophores (Deacon, 1984). Studies of

growth and nutrition of white- and brown-rot fungi in synthetic media revealed that none of the fungi required nitrogen in organic form, but growth was usually better with organic than inorganic nitrogen, such as ammonium salts (Jennison, 1952, Deacon, 1984).

Metals are directly and indirectly involved in all aspects of fungal metabolism, growth and differentiation. Trace elements, such as Fe, Zn, Cu, Mn, Mo and Ni, have an important role in metalloenzymes (Dedyukhina & Eroshin, 1991). Iron, which is of fundamental importance to living cells, exists in nature predominatly in the ferric(III) oxidation state, which is not readily available for assimilation. In wood material iron is usually tightly attached to the cell wall strucutres which, furthermore, hinders its utilisability by microorganisms. Thus, to solubilize and sequester ferric iron many filamentous fungi release high-affinity Fe-binding molecules, siderophores and organic acids (Gadd, 1993, Fekete et al., 1989, Schmidt et al., 1981, Srinivasan et al., 1994). Calcium has been observed to have an essential role in the regulation of the metabolism of the eucaryotic organisms (Highley, 1989). Materials rich in calcium, such as insulation wools and concrete, have been shown to promote the growth and decay activity of brown-rot fungi, especially of *S. lacrymans* (Bech-Andersen, 1985 and 1987, Paajanen et al., 1994). It has been suggested that *S. lacrymans* absorbs calcium into cells in order to regulate the pH conditions. Brown-rot fungi typically produce extracellular organic acids into the growth medium which might lead to excessively acidic conditions (Bech-Andersen, 1987). The amount and activity of wood polysaccharide degrading enzymes was significantly decreased by the inhibition of a calcium binding protein, calmodulin. In some cases the mycelial growth totally ceased due to this inhibition (Highley, 1989).

Thiamine, the only vitamin essential for growth of most wood-decay fungi, is sufficiently present in wood (Jennison, 1952, Highley 1987a). Thiamine is needed mainly because of the pyridine unit, which acts as a necessary precursor for the biosynthesis of thiazole compounds (Deacon, 1984).

1.3 DEGRADATION OF WOOD POLYSACCHARIDES

1.3.1 Oxidative degradation of cellulose

1.3.1.1 Production of oxidative decay agent by decay fungi

Basidiomycetous brown- and white-rot fungi occur in the same taxonomic groups. In some genera (e.g. *Poria*) it is possible to find species which belong either to brown-rotters or to white-rotters (Enoki et al., 1988). Brown-rot fungi, however, differ from other cellulolytic microorganisms in lacking the ability to hydrolyze crystalline cellulose enzymatically (Highley et al., 1983b, Highley

et al., 1987a, Knowles et al., 1987, Enoki et al., 1988). At the initial stage of brown-rot decay the degree of polymerization of cellulose (DP) decreases rapidly approximately from 10^4 to 250 before measurable signs of weight loss, bringing about reduced strength properties of wood material, can be observed (Cowling 1961, Kirk et al., 1991 Kleman-Leyer et al., 1992). The degradation mechanisms for wood polysaccharides of brown-rot fungi are still fairly unknown but it has been suggested that they possess both an oxidative and a hydrolytic pathway (Highley, 1977, Highley, 1987a, Enoki et al., 1989). The fundamental difference between white- and brown-rot decay lies in the mechanisms where the action of brown-rot fungi is directed towards the whole cellulose microfibrils, whereas white-rot fungi attack the surfaces of the microfibril producing progressive erosion (Kleman-Leyer et al., 1992).

The chemical and structural analysis from the brown-rotted wood suggests that these fungi produce extracellular compounds able to penetrate deep into the wood cell wall structures and participate in degradation reactions. These compounds break the bonds between the fibril structures and depolymerize wood polysaccharides, causing only a limited weight loss at the initial stage of decay (Highley et al., 1985, Murmanis et al., 1987, Enoki et al., 1990, Messner et al., 1984). Observations obtained by TEM microscopy lead to the suggestion that these compounds penetrate to cell wall structures along the micro pores by breaking the glucosidic bonds of polysaccharides far from the hyphae (Highley et al., 1983a). Unlike white-rot and soft-rot, in which the degradation of cell wall takes place close to the fungal mycelia, the impact of brown-rot is wide-ranged due to these diffusible degradation agents (Highley et al., 1983b). Alterations in the degree of polymerization of cellulose and the hygroscopicity of wood itself also suggest (the existence of) the action of diffusible molecules (Highley et al., 1983a). While penetrating into the wood cell wall, brown-rot fungal hyphae are often observed to be associated with an extracellular gelatinous sheat (Micales, 1989). This glucan sheat, which lines the interior of the wood cells, is thought to be important in attaching the hyphae to substrates, storing nutrients, protecting the fungus from desiccation and other environmental stresses, and concentrating and distributing degradative enzymes (Micales et al., 1989).

It has been suggested that oxidative reactions participate in the degradation of crystalline cellulose in brown-rot decay (Highley, 1977, Enoki et al., 1990). The formation of glycerin and erytronine acids as degradation products indicates the oxidative breakdown of the internal carbon bonds located in glycosyl units (Kirk et al., 1989). It has been suggested that the diffucible molecule is non-enzymatic, since the polysaccharidases produced by Basidiomycete fungi are too large to be able to penetrate into the wood cell wall structures. Cellulases have strong affinity for the substrate, thus, the effect of enzymes is local, not widespread at the initial stage of brown-rot decay (Highley et al., 1983a, 1983b). The initiation of the degradation of crystalline cellulose may be caused by the production of extracellular hydrogen peroxide. Hydrogen peroxide is a small molecule

capable to penetrating into the wood cell structures (Koenigs 1972a, 1972b, 1974a, 1974b, Highley, 1982).

The oxidative degradation of polysaccharides has been suggested to take place by the action of hydroxyl radicals which break the glycosidic bonds between the chains (Halliwell, 1965, Gilbert et al., 1984). In the studies of the degradation of lignin by white-rot fungi it has been observed that in addition to enzymes, hydrogen peroxide, hydroxyl and other oxygen radicals participate in the degradation process (Tanaka et al., 1991, Tanaka et al.,1992, Highley & Murmanis, 1985, Highley, 1987b). These compounds can also have an important role in brown-rot decay (Illman et al., 1988a, 1988b, Highley, 1987c, Backa et al., 1992, Hyde & Wood, 1995a, 1995b). Highley (1977) observed that the end products of brown-rotted cellulose consisted mainly of carbonyl and carboxyl groups, indicating the participation of radical reactions. Fenton´s reaction is an analogous radical producing system. The main principle of Fenton´s reaction is presented in the following reaction equal:

$$Fe^{2+} + H_2O_2 \text{ ---------- } Fe^{3+} + HO^{\cdot} + HO^{-}$$
$$Fe^{3+} + H_2O_2 \text{ ---------- } Fe^{2+} + O_2^{-} + 2H^{+}$$

The ability of brown-rot fungi to produce extracellular hydrogen peroxide was demostrated by Koenigs (1972a, 1974a). According to Fenton´s reaction the hydrogen peroxide produced by fungi oxidized the two valenced transition metals (Fe^{2+}, Mn^{2+}, Co^{2+}). Koenigs (1974a) observed that the concentration of iron in wood is sufficient for the reaction. Fenton´s reaction starts a series of chemical reactions producing free oxygen radicals as the end result. As highly reactive compounds, these radicals may initiate the oxidative degradation of cellulose (Highley, 1982, Highley & Murmanis, 1985, Illman et al. 1988b, Micales & Highley, 1988). The changes in the brown-rotted holocellulose have been observed to resemble changes in cellulose structure, caused by treatments with Fenton´s reagent (Cowling, 1961, Halliwell, 1965, Kirk et al., 1991).

The production of hydroxyl radicals during the oxidative degradation of cellulose by brown-rot fungi, *Tyromyces palustris* and *Gloeophyllum trabeum* was observed by Enoki et al. (1990, 1991). Their studies suggest that an extracellular substance that produces H_2O_2 and reduces H_2O_2 to hydroxyl radicals is involved in the degradation of cellulose and lignin in wood. These kinds of extracellular compounds have been isolated from the wood-containing cultures of both brown-rot and white-rot fungi. The compounds were identified as low molecular weight glycopeptides requiring H_2O_2 for one-electron oxidations. They catalyzed the redox reactions between an electron donor, such as NADH, and O_2 to produce H_2O_2 via oxygen radicals and hydroxyl radicals by Fenton´s reaction between the ferrous iron bound to the ligands and H_2O_2 (Fig. 3). The degradation of cellulose by brown-rot fungi was indirectly studued by oxidation of keto-4-thiomethylbutyric acid (KTBA) (Enoki et al., 1989).

The one-electron oxidation of KTBA occurred trough the formation of hydroxyl radicals produced by the Fenton-Haber-Weiss reaction. This leads to the suggestion that the fungal preparations used were able to reduce H_2O_2 to hydroxyl radicals (Enoki et al., 1989, 1990, 1991, Tanaka et al., 1991, 1992, Itakura et al., 1994). Backa et al. (1992, 1993) observed that on wood chips the production of hydroxyl radicals was initiated when the white-rot and brown-rot fungi started to decompose the wood material in order to attack the cellulose. According to Backa et al., (1992, 1993) the formation of hydroxyl radicals is a site-specific process involving a transition metal ion-catalyzed reduction of H_2O_2 which could be originated from the disproportation of oxygen radical anions or catalyzed by the action of oxidative enzymes.

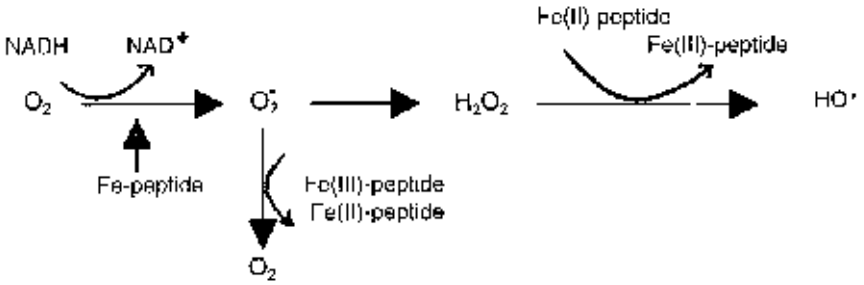

Fig. 3. Proposed mechanism for generation of activated oxygen species by glycopeptides produced by decay fungi (from Enoki et al., 1991).

Kremer & Wood (1992b) have provided the first direct evidence of the Fenton reaction by isolating cellobiose dehydrogenase (CDH) (EC 1.1.3.25) wood-rotting fungi. This enzyme was purified from white-rot and brown-rot fungi (*Phanerochaete chrysosporium* and *Coniophora puteana*). It oxidized the reducing end of cellodextrins, with a substrate size ranging from cellobiose to microcrystalline cellulose. It was proposed that the primary function of this enzyme is to act as a Fe(III) reductase, as was recently proved by kinetic calculations. In the precense of Fe(III), CDH could provide a biological method for distrupting the crystalline structure of cellulose (Kremer & Wood, 1992a, Kremer & Wood, 1992b, Kremer & Wood, 1992c, Wood & Wood, 1992, Wood, 1994, Hyde & Wood, 1995a, Hyde & Wood, 1995b). The involvement of OH radicals in cellulose breakdown creates a range of oxidized sugars. The succesful use of Fenton reaction by a living organism requires a spatial separation between initiating enzymes and the site of production of OH radicals. Owing to the high and indiscriminate reactivity of OH radicals, they have a

very short lifetime and the reaction takes place very close to their formation (Wood, 1994). According to the hypothesis of Kremer & Wood (1992a, 1992b, 1992c) at a very acidic pH, which is typical to brown-rot fungi, Fe(II) will be present as free Fe^{2+}, which is not autooxidable. Electron exchange with Fe(III) will lead to a net diffusion of Fe(II) away from hyphae, into regions of higher pH. Thus, the critical combination of Fe(II) and H_2O_2, due to the reduction of dioxygen by CDH, will be formed at a distance, while the hyphae are protected by the low local pH (Fig. 4) (Hyde & Wood, 1995b). Backa et al. (1992, 1993) also observed that the formation of hydroxyl radicals, which are highly destructive compounds, occured in a juxtaposition to lignin or cellulose, and thus should not be deleterious to the growing fungal hyphae.

Low molecular weight, high-affinity iron-binding biological chelators, siderophores, have been recently observed to be produced by white- and brown-rot fungi. The fungal hyphae probably scavenge the transition metals by the action of siderophores to be used in fungal metabolism and production of enzymes. Because of the low molecular weight of the siderophore-metal complex (below 1000 Da) and the oxidizing potential of the bound transition metals, certain siderophore structures (phenolates) may also play a potential role in the early stages of cellulose depolymerization by brown-rot fungi. Phenolate compounds isolated from brown-rot fungus *Gloeophyllum trabeum* were observed to be able to reduce Fe(III) to Fe(II) and produce hydroxyl radicals in the presence of H_2O_2. Furthermore, the ability of the phenolate siderophores to penetrate into the wood cell wall has been shown (Goodell et al., 1995, Jellison et al., 1990, 1991a, 1991b, 1995). Iron, as a compound complexed to organic acids and hydrocarbons existing in culture medium, is able to trigger to the Fenton´s reaction (Lundborg, 1989a). However, according to ESR spectroscopy studies carried out by Illman et al. (1988a, 1988b), no changes in the low-spin iron could be detected during brown-rot decay. On the other hand, the paramagnetic changes associated with manganese were observed in the early stages of brown-rot decay. The increases in the Mn^{2+} signals correlated with the wood's susceptibility to brown-rot decay possibly due to chelation of the metal (Illman et al., 1988a, 1988b).

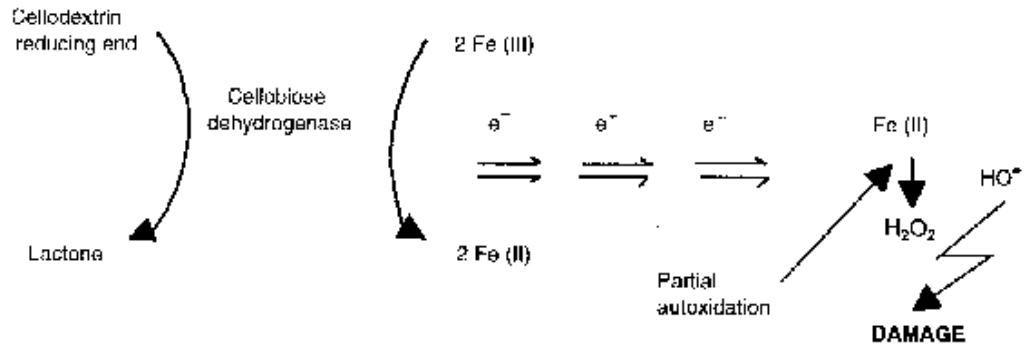

Fig. 4. Cellobiose dehydrogenase (CDH) as an agent for hydroxyl radical production in white- and brown-rot fungi (from Wood, 1994).

1.3.1.2 Production of oxalic acid by decay fungi

Both brown-rot and white-rot fungi have been shown to accumulate oxalic acid and other organic acids in the culture media (Cowling, 1961, Bech-Andersen, 1987, Green et al. 1991, Connolly & Jellison, 1994, Hyde & Wood, 1995a, 1995b) rown-rot fungi contain at least two different oxalic acid producing enzyme, glyoxylate oxidase (dehydrogenase) and oxalate hydrolase, and the production of oxalic acid is conneted to the TCA-cycle (Shimada et al., 1991). The brown-rot fungus *Tyromyces palustris* catalyses the hydrolysis of oxaloacetate yielding oxalate as product. It has been suggested that oxalic acid plays a central role the brown-rot breakdown of cellulose (Akamatsu et al., 1991, Espejo & Agosin, 1991). White-rot fungi produce oxalate decarboxylase activity (ODC) to convert oxalate further to formate and carbon dioxide (Dutton et al., 1993a, 1993b, Akamatsu & Shimada, 1993). Micales (1994) has recently reported the presence of oxalate decarboxylate also in brown-rot fungi. In the biodegradation of lignin, Shimada (1995) has observed that the enzymatic decomposition of oxalate produced by *Phanerochaete sordida* yields superoxide species under aerobic conditions. It has been reported that oxalic acid itself can also yield reduced viscosity of wood pulp and cotton cellulose, and depolymerized of hemicellulose (Bech-Andersen, 1987, Green et al., 1991, Shimada et al., 1991). The role of oxalic acid, however is still unknown. This acid may participate in the reduction of transition metals prior to the initiation of the Fenton reaction (Fig. 5) (Koenigs 1972a, 1974a, 1974b, Schmidt et al., 1981, Espejo & Agosin, 1991).

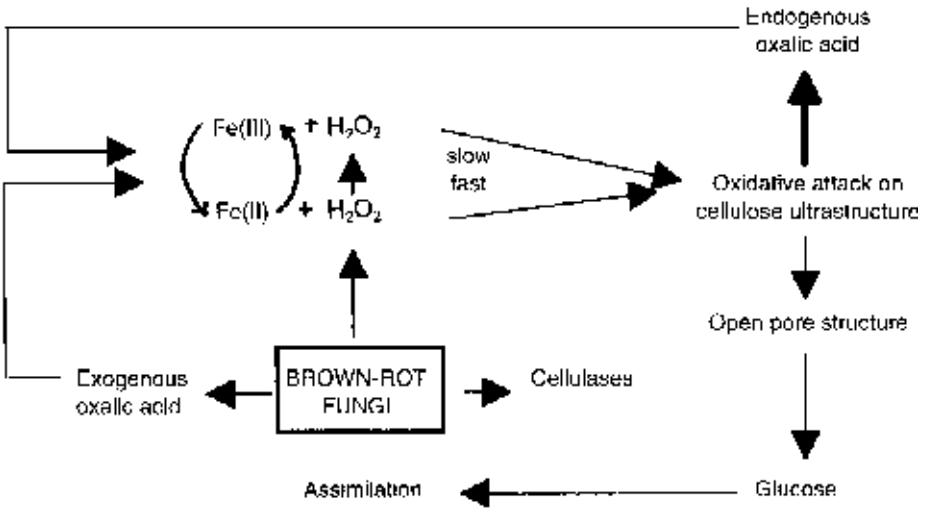

Fig. 5. The hypothetical role of oxalic acid in the brown-rot decay mechanism (from Schmidt et al., 1981).

1.3.2 Hydrolytic degradation of cellulose

1.3.2.1 Enzymatic degradation of crystalline cellulose

In most natural sources, cellulose is embedded in other cell wall components such as hemicelluloses and lignin. The solubilization of native cellulose threfore represents a major challenge to microbes (Teeri et al., 1992). Wood degrading microorganisms produce a wide range of extracellular hydrolytic enzymes. The degradation of cellulose by the hydrolytic action of cellulases is affected by several factors, such as type of enzymes, quality of substrate and environmental factors (temperature, pH etc.) (Highley, 1988, Sanyal et al., 1988). Cellulases, containing several isoenzymes, are classified into three main groups accordign to the mode of action and biochemical structure of the protein (Table 1) (Lappalainen, 1988, Eriksson et al., 1990).

As a physically complex material cellulose is made up of highly ordered crystalline and disordered amorphous regions (Fengel & Wegener, 1989). In 1950´s Reese & Levinson (1952) suggested that the hydrolytic degradation of cellulose was due to the synergistic action of two different enzyme complexes, C_1 and C_x. The crystalline cellulose was thought to be hydrolysed by the C_1 activity and the amorphous cellulose by the C_x-complex.

It is now known that the activity of endoglucanases (EG), cellobiohydrolases (CBH) and β-glucosidases is needed for the complete hydrolysis of crystalline cellulose (Nevalainen & Penttilä, 1995). The cellulolytic system of the mesophilic soft-rot fungus, *Trichoderma reesei*, is well-known. Several enzymes needed for the breakdown of crystalline cellulose are produced by this organism. The enzymatic degradation of cellulose is still not fully understood. It was previously assumed that the synergistic hydrolysis of cellulose was initiated by the action of endoglucanase (EG) liberating reducing ends of the cellulose chains for the action of cellobiohydrolases (CBH). The cellobiose units formed were then hydrolyzed to glucose by the action of β-glucosidase. The hydrolysis results obtained by the use of pure enzyme preparates, however, have provided evidence of the initial role of CBH activity in the hydrolysis of native cellulose (Enari & Niku-Paavola, 1987). It has been shown that cellulases act in synergy during cellulose hydrolysis (Fig. 6). It is generally considered that endoglucanases randomly attack β-1,4-glucosidic linkages in the amorphous parts of cellulose and also hydrolyze barley β-glucan and substituted celluloses such as carboxymethyl (CMC) and hydroxyethyl (HEC) cellulose. Cellobiohydrolases do not hydrolyze substituted celluloses; they are mainly responsible for the hydrolysis of crystalline cellulose (Nevalainen & Penttilä, 1995). Synergism enables an effective hydrolytic process (Knowles et al., 1988, Lappalainen, 1988). In addition to the synergistic action of EG and CBH complexes, the action of cellobiohydrolases has also been observed to be synergistic (Henrissat et al., 1985, Fägerstam & Petterson, 1986, Teeri, 1987, Wood et al., 1990).

As result of the action of CBH, the cellotriose is first produced through a condensation reaction of the CBH between the hemiacetal-OH of β-D-cellobiose and the C_4-carbinol of the non-reducing terminal of an other cellobiose molecule (Okada & Tanaka, 1988). The cellotetraose formed is split into cellotriose and glucose as well as two molecules of cellobiose. The cellotriose is subsequently split into cellobiose and glucose (Uemura et al., 1993). The cellobiose units are then split to monomeric sugars (glucose) by β-D-glucosidase activity (EC 3.2.1.21) (Jeffries, 1987, Knowles et al., 1987, Sanyal et al., 1988, Eriksson et al., 1990). The effect of the degree of cellulose polymerization on the binding specificity of the enzyme and the affinity for long substrate chains form the most important classifying criteria for cellulases (Jeffries, 1987). The cellulolytic enzymes are usually induced as multi enzyme complex consisting of five or more enzymes (Knowles et al., 1987).

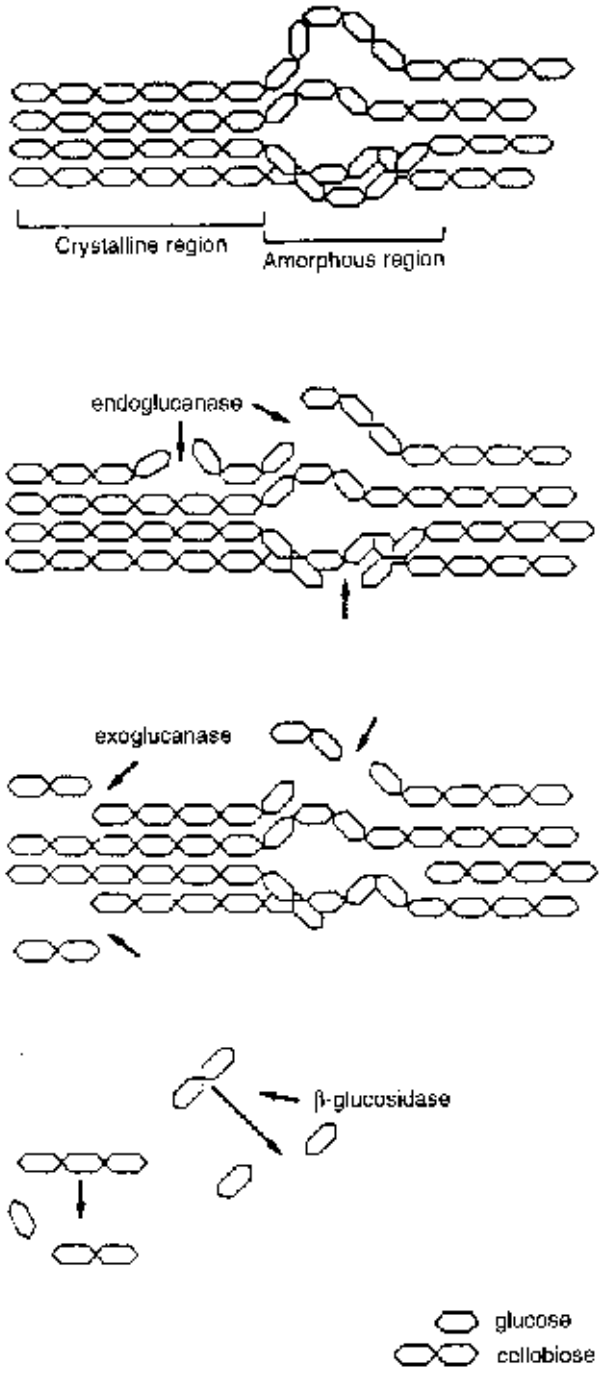

Fig. 5. The synergistic hydrolyzation of crystalline cellulose (from Nevalainen & Penttilä, 1995).

Table 1. The classification of cellulase complexes.

Systematic name	Number	Trivial name	Substrate	End product
1,4-β-D-glucan-cellobiohydrolase	EC 3.2.1.91	Exoglucanase, Cellobiohydr-olase	Crystalline cellulose	Cellobiose
Endo-1,4-β-D-glucan-4-glu-canohydrolase	EC 3.2.1.4	Endoglucanase, β-glucanase	amorphous cellulose	cellooligo-saccahrides
β-D-glucosidic-glucohydrolase	EC 3.21.21	Cellobiase, β-glucosidase	cellobiose, cellotriose	glucose

Only a few microorganisms, mainly members belonging to the genera of Ascomycetes and Deuteromycetes (e.g. *Trichoderma* sp., *Fusarium* sp., *Penicillium* sp.) possess a complete cellulolytic system. Of the Basidiomycetous fungi some white-rotters are able to produce a complete hydrolysis of cellulose (Selby, 1969, Wood, 1975, Eriksson et al., 1990, Wood et al., 1990).

1.3.2.2 The cellulolytic system of brown-rot fungi

Comparatively little is known about of the collulolytic system of brown-rot fungi. Degradation of cellulose by brown-rot fungi differs greatly from other cellulolytic microorganisms, such as *Phanerochaete chrysosporium* and *Trichoderma reesei*. Most brown-rot fungi are able to degrade enzymatically amorphous cellulose or subsituted cellulose substrates (eg. carboxymethyl cellulose, and hydroxyethyl cellulose), but these fungi are supposed to be unable to degrade crystalline cellulose by the synergistic action of endo- and exoenzymes (Nilsson, 1974, Highley, 1973, Highley, 1976, Highley, 1977, Highley, 1987b, Highley, 1988, Micales & Highley, 1988).

Brown-rot fungi produce measurable amounts of endo-1,4-β-glucanases (EC 3.2.1.4) and β-glucosidases. The extracellular enzyme preparations obtained from these fungi have not been observed to be able to degrade crystalline cellulose (Uemura et al., 1993, Highley, 1982, Micales et al., 1989, Micales & Highley, 1988). The endoglucanases isolated from the brown-rot fungus *Meruliporia* (*Serpula*) *incrassata* preferentially cleaved cellulose microfibrils at the amorphous sites, leaving behind predominantly crystalline cellulose (Kleman-Leyer & Kirk, 1994). Schmidhalter & Canevascini (1992) have found some evidence for the production of cellobiosehydrolase activity by the brown-rot fungus *Coniophora puteana* in a culture medium containing amorphous cellulose as carbon source. This exo-acting cellulase was detected by agluconic bond cleavage of 4-methylumbelliferyl-β-D-cellobioside or p-nitrophenyl-β-D-lactoside. Interference of β-glucosidase was eliminated with glucono-δ-lactone and the reaction was strongly inhibited by cellobiose. The difference in the cross-reactivity of

cellulolytic enzyme systems of brown-rot and white-rot fungi with the polyclonal antibodies to the CBH I was studied by enzyme-linked immunosorbent assay (ELISA). The enzymes system from brown-rot fungi, however, gave negative response towards antibodies from white-rot enzymes. This suggests the absence of the homologous sequences and structures of CBH I in brown-rot fungi (Uemura et al., 1993).

Typically to brown-rot decay a modified lignin residue is produced. Brown-rot fungi have not been observed to possess enzyme activities needed for the degradation of lignin (Jin et al., 1990a, Kim & Newman, 1995, Newman & Kim, 1995). However, recently the brown-rot fungus *Polyporus ostriformis*, was reported to form lignin peroxidase (Dey et al., 1991). These findings may suggest that the enzymatic machinery of brown-rot fungi might resemble, at least in some instances, those of white-rot fungi (Schmidhalter & Canevascini, 1992).

1.3.3 Degradation of hemicellulose

Hemicellulases are classified according to their substrate specificity. Hemicellulases form a group of enzymes which consists several endo- and exoglucanases and esterases able to hydrolyze the backbone chains and side-groups of the polysaccharide releasing sugar and acid units. The production and properties of endo-β-1,4-xylanase, endo-β-1,4-mannanase, β-D-xylosidase, β-D-mannosidase, α-arabinosidase, α-galactosidase, α-glucuronidase and acetylesterases have been studied from many microorganisms (Poutanen, 1988, Wong et al., 1988, Eriksson et al., 1990).

Xylanases are produced by many species of bacteria and fungi. Xylanases of *Aspergillus niger* and *Trichoderma* spp. have been studied and characterized most extensively (Poutanen 1988, Wong et al., 1988). Most xylanases are rather small (around 20 kDa) monomeric proteins (Viikari et al., 1993). Xylanases are classified according to their chemical and physical properties. The 1,4-β-D-xylopyranose bonds of D-xylan are hydrolyzed randomly by the activity of 1,4-β-xylane xylanohydrolase (endoxylanase, EC 3.2.1.8). Exo-1,4-β-D-xylosidase (EC 3.2.1.37) catalyzes the hydrolysis of 1,4-β-D-xylans by removing successive D-xylose residues from the non-reducing termini (Poutanen, 1988). Fungi are found to possess only endoxylanases. Most xylanases do liberate xylose during the degradation process but, only β-xylosidases have been shown to have real xylobiase activity. Of the structural components forming hemicellulose, xylan has the main role for cohesion of fibres (Lappalainen, 1988, Cavazzoni et al., 1989, Khowhala et al., 1988, Micales et al., 1987, Rypacek, 1977, Wong et al., 1988).

Endo-1,4-β-mannanases (EC 3.2.1.78) catalyze the random hydrolysis of the β-D-1,4-mannopyranosyl linkages within the main chain of mannans and various polysaccharides consisting mainly mannose. The production of mannanases by fungi is usually induced by mannans, e.g. locust beam

gum, or by cellulose, which has been observed to be an effective mannanase inductor (Johnsson, 1990, Viikari et al., 1993).

The enzymes taking place in the degradation of lignocellulose form enzyme complex which consist of several enzymes having their own specialized tasks to ensure the effective hydrolysis. It has been found that most of the enzymes taking part in the decomposition of lignocellulose (e.g. xylanases) have overlapping specificity. This makes it possible to maximize hydrolysis of the substrate (Teeri, 1987, Wong et al., 1988).

1.4 AIM OF PRESENT STUDY

The brown-rot degradation mechanisms are still fairly relatively little-known despite vigorous research during past few decades. An undestanding of the initial reactions is of extreme practical importance for the development of new, environmentally safer wood preservation methods. The aim of this study is to clarify the reactions taking place in the initial degradation of wood polysaccharides by brown-rot fungi. The main interest focuses on understanding the initial reactions taking place in the oxidative degradation of wood polysaccharides. The production and induction of extracellular hydrogen peroxide, which is suggested to be the small molecular size, diffusible compound participating in the degradation of cellulose, and oxalic acid was studied. The production of cellulases and hemicellulase was studied in order to clarify the role of hydrolytic enzymes in brown-rot decay. Biomimetic studies of the action of Fenton´s reaction were carried out. The results obtained were used to produce hypothetical model describing the biochemical events in brown-rot decay in order to clarify the key-step reactions essential for future wood preservation.

2 MATERIALS AND METHODS

2.1 CULTIVATION METHODS

2.1.1 Fungal strains and precultivation

The brown-rot strains used in the present investigation were *Poria placenta* (FRLP 208), *Gloeophyllum trabeum* (BAM Ebw. 109), *Coniophora puteana* (BAM Ebw. 15) and *Serpula lacrymans* (Sl 1), obtained from the VTT Building Technology Wood Technology collections. The strains were precultivated on solid malt extract medium (5 %) for 1-2 weeks at 20 ^{o}C in the dark.

2.1.2 Cultivation conditions for the production of H_2O_2 for qualitative analysis (I)

The wood based culture medium consisted of fine spruce sawdust (*Picea abies*) (4 g) with graham flour (40 mg) as starter carbohydrate per each petri dish. The medium was moistened with 40 ml distilled water with 1.5 % agar.

The production of hydrogen peroxide on artificial culture media was detected by *P. placenta* and *S. lacrymans*. The solid culture media used was developed by Highley (1976) for brown-rot fungi. The carbon sources used in a concentration of 1 % were microcrystalline cellulose (Avicel, Serva), β-glucan (Biocon), xylan (beech wood xylan), Kraft lignin (Indulin, Westvaco), glucose (Merck), xylose (Sigma) and cellobiose (Sigma).

The sterilized (autoclave; 120^{o}C, 20 min) culture media were inoculated with the test fungi using a small agar slant with mycelia taken from malt extract plates. The incubations were carried out at 20^{o}C in the dark.

2.1.3 Cultivation conditions for the production of H_2O_2, oxalic acid and endoglucanase for quantitative analysis (IV)

The fungal strain used was *Poria placenta* (FRLP 208). The induction of hydrogen peroxide and oxalic acid production was studied in liquid culture media with three different carbon sources: 1 % microcrystalline cellulose (Avicel), 1 % amorphous cellulose (phosphoric acid swollen Avicel) (Walseth, 1952) and 0.5 % glucose (IV). The cultivations were carried out in 250-ml Erlenmayer flasks containing 50 ml liquid basal salts (2.0 g NH_4NO_3, 2.0 g KH_2PO_2, 0.5 g $MgSO_4$ x 7 H_2O, 0.1 g $CaCl_2$ x 2 H_2O, 0.001 g thiamine hydrochloride, 0.036 g $MnCl_2$ x 4 H_2O, 0.31 g $ZnSO_4$ x 7 H_2O, 0.039 g $CuSO_4$ x 5 H_2O, 0.0018 g $(NH_4)_6Mo_7O_{24}$ x 4 H_2O per 1000 ml distilled water) and 1 % cellobiose as starter carbohydrate (Highley, 1976). The test fungus was first inoculated in small malt extract agar slants 1 % cellobiose medium (Highley, 1976) and after 2 weeks the culture was homogenized and 10 % (v/v) cellobiose inoculum was transferred to the production media. The pH of the culture media was adjusted to 3.5 prior to the inoculation. The culture media were sterilized by autoclave. Each cultivation had two replicates and the cultivations were carried out at 20 ^{o}C.

2.1.4 Cultivation conditions for the production of hydrolytic enzymes

The test fungus used was *Gloeophyllum trabeum* (BAM Ebw. 109). The production of hydrolytic enzymes was carried out in liquid, wood based culture medium consisting of 1 % spruce sawdust with 0.1 % graham flour in distilled water and in liquid medium containing 1 % microcrystalline cellulose (Avicel) as carbon source. The culture media were autovlaved prior the inoculation. The test fungus was inoculated in small malt extract

agar slants. All the cultivations were carried out in 250-ml Erlenmayer flasks with 50 ml medium on an incubation shaker at 22 ^{o}C in the dark (II).

The production of xylanase was carried out in an air-lift reactor with a 1.2/l working volume at 37 ^{o}C for 9 days (III). The culture medium for the enzyme production contained 10 g/l spruce sawdust with 0.01 g/l graham flour as a starter carbohydrate in distilled water. The culture medium was autocalved prior to inoculation. The culture medium was inoculated with homogenized mycelium of the test fungus grown on malt extract medium.

2.2 BIOCHEMICAL ANALYSIS

2.2.1 Qualitative and quantitative detection of hydrogen peroxide

The extracellular hydrogen peroxide produced by test fungi was qualitatively measured by using horseradish peroxidase (HRP) (Sigma, 1280 units/mg solid) and 2,2-azinobis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS, Sigma) (Muller, 1984, Highley, 1987c) (I). The reaction mixture (0.5 ml per test plate), containing 25 μg of HRP and 200 μg of ABTS was added to the solid agar plates (20 ml culture medium per plate). The ability of test strain to produce extracellular H_2O_2 was detected visually during incubation as the formation of green colour around the youngest part of mycelia. The plates with 0.5 ml of ABTS without HRP were used as controls.

The production of hydrogen peroxide was quantitatively detected by the method of Highley (1987c) as a function of time (IV). The pH of the culture media was adjusted to 5 by adding 5 N NaOH before the detection. 6 μl of a mixture of ABTS and HRP was added to a 220 μl sample of culture filtrate. The reaction mixture contained 20 μg/μl of ABTS and 4 μg/μl of HRP. In the reference sample HRP was omitted. The absorbances of both samples were measured at 410 nm after a reaction time of 2 minutes at 20 ^{o}C. The amount of hydrogen peroxide (μmol/l) was calculated according to standard curve from the difference of the absorbances ($A_{(ABTS\text{-}HRP)}$-$A_{(ABTS)}$).

2.2.2 Detection of oxalic acid

Oxalic acid produced by *P. placenta* was detected by using a diagnostic oxalate kit according to manufacturer´s instruction (Boehringer Mannheim) (IV). The method is based on the cleavage of oxalic acid to formic acid in the presence of oxalate decarboxylase. The formate is further oxidized to bicarbonate by NAD in the presence of formate dehydrogenase (FDH). The amout of NADH formed during the reaction is stoichiometric with the amount of oxalic acid. The amount of oxalic acid produced was checked by HPLC. The detection was carried out in Millipore/Waters anion column with lithium borate gluconate buffer as eluent.

2.2.3 Sugar analysis

The realesed sugars in the culture media were analysed by HPLC using an HC-40 column (Hewlett-Packard, 1090 LC-system). Pure glucose and cello-oligosaccharides (Fluka): cellobiose, cellotriose and cellopentaose were used as standars (IV).

2.2.4 Enzyme assay methods

Before the analysis of hydrolytic enzymes the culture filtrates were concentrated by vacuum evaporation at 50 ^{o}C. Endo-β-1,4-glucanase, endo-β-1,4-xylanase, β-xylosidase, endo-β-1,4-mannanase, β-glucosidase and total cellulase (FPU) activities were measured by the methods of Mandels et al.,(1976), Bailey et al., (1992), Stålbrand et al. (1993) and IUPAC (1987) (II - IV). The reaction mixture contained 0.05 M sodium citrate buffer, pH 5.3 (1,8 ml), the substrate (1 %) and the concentrated culture filtrate (200 µl). The substrates used in these measurements were hydroxyethyl cellulose (HEC), beech wood xylan, p-nitrophenyl-b-D-glucopyranoside, locust bean gum and filter paper (Whatman no. 1). 1,4-β-xylosidase (EC 3.2.1.37) activity was assayed in 50 mm Na-citrate buffer, pH 4.0 by using 5 mM p-nitrophenyl-b-xylopyranoside as substrate at 50 ^{o}C (Poutanen & Puls, 1988). The incubations were carried out at 50 ^{o}C. The activies of endoglucanase, xylanase, xylosidase, glucosidase and mannanase were expressed as nanokatals and total cellulase activity was expressed as IU.

2.2.5 Protein assay methods

Soluble proteins were assayed according to Lowry et al. (1951) (III & IV) or with a commercial test solution (Bio-Rad Protein Assay, Bio-Rad Laboratories) according to Bradford (1976) (II). The amount of extracellular proteins was measured directly from the culture liquid after concentration by vacuum evaporation. For the measurements of mycelial protein content the proteins were extracted from the homogenized mycelia by boiling the mixture for 5 min in 1 N NaOH. The mixture was neutralized and the proteins were precipitated with cold aceton over night (Herbert et al., 1971) (IV).

2.2.6 Purification of the xylanase (III)

The culture filtrate (850 ml) was concentrated to 50 ml by vacuum evaporation at 20 ^{o}C and dialyzed overnight against 10 mM sodium phosphate buffer, pH 6.5, at 4 ^{o}C. The buffered samples (15 ml at time) were applied to an anion-exchange column of DEAE-Sepharose FF equilibrated with the same buffer. The unbound proteins were washed with 10 mM sodium phosphate buffer, pH 6.5, containing 50 mM of NaCl. The bound proteins were eluted from the column with a gradient of 50-200 mM NaCl. The pooled and concentrated xylanase fraction was buffered to pH 7.1 with 25 mM imidazole-HCl buffer and applied to a chromatofocusing PBE 94 column (Pharmacia) and eluted with Polybuffer-HCl, pH 4.0 (Phar-

macia) according to manufacturer´s instructions. For further characterization of the enzyme, the pooled fraction was concentrated by vacuum evaporation to 5 ml and buffered with 10 mM sodium phosphate buffer, pH 6.5, containing 0.2 M NaCl and applied to a pre-equiliberated (10 mM sodium phosphate buffer, pH 6.5) Sephacryl S-100 HR gel filtration column. The enzyme was eluted with the same buffer with a linear flow rate of 14.6 cmh^{-1}. The purified enzyme was stored at -20 ^{o}C.

The molecular weight of the xylanase was determined under denating conditions by using 12.5 % polyacrylamide gel slab in a Phast-system according to the manufacturer´s instructions (Pharmacia). The low molecular weight calibration mixtures (Pharmacia) were used as standards. Before electrophoresis, the protein sample was further concentrated by lyophilization. The M_r of the native xylanase of *G. trabeum* was also measured by gel filtration in Sephacryl S-100 gel. The column was calibrated with low M_r Pharmacia protein standards (13 700-67 000) and aldolase (158 000). The void volume was determined with blue dextran (Pharmacia/LKB).

2.2.7 Analysis of the physical properties of purified xylanase (III)

The effect of pH of the xylanase activity was measured in following buffers: 50 mM sodium citrate buffer at pH 4 - 7 and 50 mM Tris-HCl buffers at pH 8 - 9. The stability of the enzyme was determined after incubating the enzyme in the buffers described above for 2,4 and 24 h at room temperature and by measuring the residual activity. The effect of temperature on the xylanase activity was determined in 50 mM sodium citrate buffer, pH 4, at 40, 50, 60, 70, 80 and 90 ^{o}C for 30 min. The thermostability of xylanase was determined by incubating the enzyme solution at 40, 50, 60 and 70 ^{o}C for 2, 4 and 24 h followed by the xylanase assay.

The purified xylanase was used in hydrolysis experiments on different xylan substrates. Hydrolysis was carried out in 50 mM sodiun citrate buffer, pH 4, at 40 ^{o}C. The incubation time was 24 h. The substrates used were substituted glucuronoxylan (DMSO-xylan) prepared by dimethyl sulphoxide extraction according to Hägglund et al. (1956), deacetylated methylglucuronoxylan (Roth) and unsubstituted xylan (Lenzing) at a concentration of 0.10 %. Enzyme dosage used was 5000 nkat/g. The reduced sugars released during the incubation were measured by the DNS-method (Sumner & Somers, 1949). The hydrolysis products were also analysed by HPLC using an HC-40 column (Hewlett-Packard, 1090 LC-system). The flow rate was 0.5 ml/h and the temperature was 80 ^{o}C. Pure xylose (Fluka) and xylooligosaccharides: xylobiose, xylotriose and xylotetraose (Megazyme) were used as standars.

2.2.8 Inhibition tests of purified xylanase (III)

The inhibition of xylanase activity was studied by incubating the enzyme for 30 min at 50 ^{o}C in 50 mM Na-citrate buffer, pH 4 containing 10 mM sodium chloride, potassium chloride, magnesium chloride, barium chloride, lithium chloride, calsium chloride, magnesium chloride and aluminium chloride. The influence of 50 mM ethylenediaminetetraaxetic acid (EDTA) and sodium tripolyphosphate (SPTT), 50 mM α-pinene, 50 mM limonene and 50 mM terpenol on the xylanase activity were also tested. After the incubation the residual xylanase activity was measured.

2.3 THE BIOMIMETIC EXPERIMENTS WITH THE FENTON REAGENTS

2.3.1 Oxidative and enzymatic treatments

The pH of substrates (microcrystalline cellulose (Avicel, Serva 14204) and spruce sawdust) in water (0.5g/50 ml) were set to pH 4.0 by using H_2SO_4, and the mixtures were incubated in a shaking water bath at 30 ^{o}C in 0.2 % H_2O_2 and 0.1 mM Fe_2SO_4 or 1 % H_2O_2 and 0.5 mM Fe_2SO_4 for 24 hours. After incubation the substrates were recovered by centrifugation, the residual H_2O_2 was removed by treating with catalase (Sigma) in 0.1 M phosphate buffer, pH 7.0 at 25 ^{o}C for 3 hours, the substrate was washed with water and suspended in 50 ml of sodium citrate buffer, pH 5.0 or sodium acetate buffer, pH 4.0 and hydrolyzed with a commercial cellulase preparation Econase (CE, Primalko), EG I (VTT/BEL) or gel-filtered *P. placenta* endoglucanase for 48 hours at 40 ^{o}C.

2.3.2 Analysis and enzymatic assays

After oxidative and enzymatic treatments the substrates were analyzed for residual dry weight. The carbohydrate composition of the spruce sawdust samples were analyzed according to Hausalo (1995). The hydrolyzates were analyzed for reducing sugars with the DNS method (Sumner & Somers, 1949). The solubilized oligomers in the Avicel hydrolyzates were analyzed by HPLC using HC-40 column (Hewlwett-Packard, 1090 LC-system) with glucose, cellobiose, cellotriose, cellotetraose and cellopentaose as standards. The solubilized oligomers from spruce were further hydrolyzed to monomers using a mixture of cellulases and hemicellulases and the sugar composition was analyzed by HPLC (Buchert et al., 1993b).

3 RESULTS AND DISCUSSION

3.1 THE OXIDATIVE DEGRADATION OF CELLULOSE BY BROWN-ROT FUNGI

3.1.1 Production of hydrogen peroxide on solid media by brown-rot fungi

In this study the ability of four brown-rot fungi (*S. lacrymans*, *P. placenta*, *C. puteana* and *G. trabeum*) to produce extracellular hydrogen peroxide was examined on a medium containing a reaction mixture of the chromogen ABTS and horseradish peroxidase (HRP) (Highley, 1987c, Muller, 1984) (I). HRP catalyses the oxidation of ABTS to a coloured compound in the presence of hydrogen peroxide. The principle of this method is presented in the following reaction equation:

$$H_2O_2 + \text{ABTS - HRP} \text{ ------- coloured compound} + H_2O$$

Two brown-rot fungal strains, *Serpula lacrymans* and *Poria placenta* were shown to be able to produce detectable amounts of hydrogen peroxide on sawdust medium (Fig. 7). The colour reaction appeared after 2 - 6 weeks incubation (I/Table 2). The test method was based on the addition of reagents trapping the hydrogen peroxide produced immediately in situ. In the absence of HRP, no reaction could be detected. However, the enzyme laccase (EC 1.10.3.2) is also able to oxidize ABTS directly to the coloured compound (Niku-Paavola et al., 1990). Thus, this method does not distinguish the production of hydrogen peroxide if laccase is present. However, without added HRP no colour appeared indicating the absence of laccase production in brown-rot fungi. Sawdust can be considered as a natural substrate for these fungi. This growth medium is very poor with respect to nutrients. Highley (1987c) has reported that the production of hydrogen peroxide by brown-rot fungi is stimulated by low nitrogen and sugar concentrations in the growth medium. In his studies the production of extracellular hydrogen peroxide was detected in 11 of 13 strains of brown-rot fungi under limited carbohydrate and nitrogen growth conditions (Highley, 1987c).

The two other brown-rot fungi, *C. puteana* and *G. trabeum*, did not produce detectable amounts of hydrogen peroxide to the sawdust medium (I/Table 2). The reasons for this may be that these fungi do not produce sufficient amounts of hydrogen peroxide for the positive reaction using the chromogen ABTS or the production of hydrogen peroxide cannot be detected under the test conditions. It is also possible that these fungi do not produce hydrogen peroxide, but instead some other small diffusible molecules which act in a similar manner to

hydrogen peroxide, generating hydroxyl radicals in the oxidative cellulose degradation pathway.

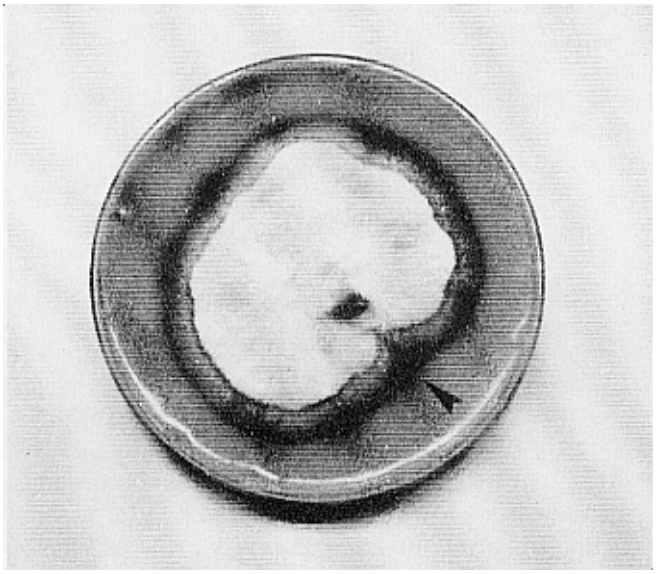

Fig. 7. The production of extracellular hydrogen peroxide by S. lacrymans on sawdust medium containing chromogen ABTS and horseradish peroxidase.

S. lacrymans and *P. placenta* were also able to produce clearly detectable amounts of H_2O_2 while growing on a culture medium containing pure cellulose (Avicel) as carbon source (I/Table 3). The colour reaction appeared after 5 - 12 days incubation in *P. placenta* and *S. lacrymans* plates. The production of extracellular hydrogen peroxide was also observed on the medium which contained beech wood xylan as carbon source. This preparation contains 75 % xylan and the substrate has only few side groups attached to the xylan backbone (Lenz & Schurz, 1986). The colour reaction appeared after 7 days' incubation on *P. placenta* plates and after 7 - 10 days' incubation on *S. lacrymans* plates. The culture media containing lignin, β-glucan, glucose or cellobiose as carbon sources did not induce the production of extracellular hydrogen peroxide by the brown-rot fungi studied. However, when xylose was used as a carbon source the production of H_2O_2 was induced on *S. lacrymans* plates, but not on *P. placenta* plates. Thus, it appears that the production of hydrogen peroxide might be induced by carbohydrates of higher degree of polymerization and in the case of cellulose, crystallinity.

The involvement of hydrogen peroxide in cellulose degradation by brown-rot fungi has been questioned because its production by brown-rot fungi has not been succesfully demonstrated in laboratory tests (Veness & Evans, 1989). A reason for this may be that the amount of secreted hydrogen

peroxide is too low for the chromogens used to detect its presence or that previously used methods may not have been sensitive enough (Highley, 1982, Highley & Murmanis, 1985). Another reason could be that the oxidative reaction is a rapid process and the hydrogen peroxide produced is consumed quickly if suitable transition metals are available (Highley, 1987c, Lundborg, 1989b). The method used in this study has proved to be a sensitive method for detection of hydrogen peroxide production (Highley, 1987c). The brown-rot fungi cannot break down lignin to low molecular weight products and lack the lignin degrading enzymes (Jin et al., 1990a). Consistent with this, lignin was not found to induce the production of hydrogen peroxide by brown-rot fungi in this study (I/table 3).

3.1.2 Production and induction of hydrogen peroxide and oxalic acid on liquid media by *P. placenta*

The production of hydrogen peroxide by *P. placenta* was clearly observed also on liquid culture media containing either amorphous (Walseth cellulose) or crystalline cellulose (Avicel) as carbon source. The highest amount of hydrogen peroxide was detected on amorphous cellulose. Accumulation began soon after inoculation, reaching a maximum in three weeks and declining sharply threafter (IV/Fig 1). The production pattern of hydrogen peroxide on crystalline cellulose was similar to that on amorphous cellulose, but the concentration was only half as much of that on the amorphous cellulose. On glucose medium, only a low amount of hydrogen peroxide could be detected after one week cultivation (Table 2).

Research on the brown-rot decay mechanism has been focused on identifying the low molecular weight compounds enhancing cellulose depolymerization at the inital stages of brown-rot decay. In this study, relatively high amounts of extracellular hydrogen peroxide were produced by *P. placenta* especially on media containing cellulose, whereas on glucose only a low amount was detected during the early stages of growth. The crystallinity of the cellulose seemed to have an effect on the production of hydrogen peroxide. The amount of hydrogen peroxide produced on microcrystalline cellulose (Avicel) was only half of that on amorphous cellulose. The production patterns were identical on both substrates. Avicel is a commercial cellulose prepared by acid hydrolysis. It consists of both crystalline and amorphous regions with an average degree of crystallinity of 45 - 60 % (Coughlan, 1992). Each culture media contained 1 % cellobiose as an additional carbon source. Cellobiose has been explained to act as an activator in the fungal degradation metabolism (Cotoras & Agosin, 1992). In this study, the addition of cellobiose to the culture media seemed to increase the amount of hydrogen peroxide produced on crystalline and amorphous cellulose. The accumulation of hydrogen peroxide on media lacking cellobiose was similar to these results, altough generally lower (results not shown).

Table 2. The effect of the carbon source (1 % microcrystalline cellulose (Avicel), 1 % amorphous cellulose (Walseth) and 0.5 % glucose) on the pH of the culture media and on the production of hydrogen peroxide and oxalic acid by P. placenta.

Culture media	Cultivation time (days)	pH	Hydrogen peroxide (μM)	Oxalic acid (g/l)
1 % Avicel	0	3.2	1.15	0
	7	2.8	7.78	0.11
	14	2.5	12.43	0.11
	22	2.3	20.30	0.27
	28	2.4	7.30	0.11
1 % Walseth	0	3.3	3.88	0
	7	3.0	21.10	0.27
	14	2.2	31.60	0.68
	22	2.2	39.50	1.00
	28	2.0	26.90	0.23
0.5 % Glucose	0	3.2	0.96	0
	7	3.0	5.50	0.05
	14	3.0	0.54	0.02
	22	2.8	0	0.03
	28	2.8	0	0.02

The production of hydrogen peroxide by *P. placenta* clearly dependent on the pH of the culture medium. On each medium the accumulation of hydrogen peroxide was preceeded by a drop in the pH of the culture medium. On amorphous and crystalline cellulose media, the pH of the culture medium rapidly decreased below 3, reaching the lowest values, 2.0 and 2.3 after four and three weeks cultivation, respectively. On glucose medium the pH seemed to be quite stable during cultivation, decreasing only from 3.2 to 2.8 (Table 2, IV/Fig. 2.). The pH decrease was mainly due to the production of oxalic acid.

In this study the most pronounced production of oxalic acid, 1.0 g/l, was observed on amorphous cellulose after three weeks' cultivation. The lowest amount was detected on glucose medium. The production of oxalic acid was ensured by HPLC, and it was found that oxalic acid was the main acid produced. The production of hydrogen peroxide and oxalic acid occurred simultaneously on crystalline and amorphous cellulose. Thus, amorphous cellulose seems to have a key role in the initiation of carbohydrate decomposition by brown-rot fungi. (Table 2, IV/Fig.3.). Green et al. (1994) have reported that *P. placenta* is able to decrease the pH to 1.7 on wood based medium within 7 d with a concomitant production of oxalic acid.

The growth of the fungus was estimated by measuring the mycelial protein content (IV/Fig. 5). As could be expected, growth was faster on amorphous

than on crystalline cellulose, which may also affect general metabolic activity and the excretion of hydrogen peroxide. It was observed that the turbid culture liquid containing amorphous cellulose cleared during cultivation, indicating changes in the cellulose structure due to enzymatic activity on fungal metabolism. On glucose medium, the protein content was surprisingly low, remaining at about 5 mg/l during the cultivation. The consumption of glucose by the cultures was very slow. The sugar analysis showed that the amount of glucose increased and the amounts of cellobiose and cellotriose decreased during the cultivation (IV/Table 1). The increasing glucose concentration originated at least partly from the starter cellobiose, but also from hydrolysis products of amorphous or crystalline cellulose. The formation of cellotriose both on glucose and cellulose based media suggest of the presence of glycosylating activity, possible by the endoglucanase.

Green et al., (1994) have suggested that substrates like hemicellulose and pectin induce the production of oxalic acid. Solubilization of hemicellulose and pectin by oxalic acid could provide rapid access for diffusible depolymerizing agents to the cellulose structure of wood cell wall. Micales (1992b) observed that the production of oxalic acid by *Postia placenta* was induced in the presence of glucuronate, which is an oxidized form of glucose, and to a lesser extent in the presence of glycolic acid and glyoxylate. Simple sugars, such as glucose, cellobiose or xylose, did not induce the production of oxalic acid. These results indicate that the biosynthesis of oxalic acid in *Postia placenta* might occur via the glyoxylate bypass of the tricarboxylic acid cycle (Micales, 1992b). In this study the accumulation of oxalic acid ceased after 3 weeks' cultivation, simultaneously with the cessation of the production of hydrogen peroxide on cellulose media. Thus, it seems that the production of hydrogen peroxide and oxalic acid decreased as a result of cessation of growth or other regulation mechanisms, and these compounds were further used in the reactions of the oxidative cellulose pathway. Cessation of the production of oxalic acid could also be due to regulation of the concentration of oxalic acid produced during fungal growth by oxalate decarboxylase (Micales, 1994).

The biochemical role of oxalic acid in the brown-rot decay is not clear. It has been suggested that oxalic acid acts as a detoxification mechanism for excess cations, such as calcium and copper, in white-rot fungi and has a role in lignin degradation (Dutton et al., 1993a, 1993b). Most brown-rot fungi, however, have been observed to produce oxalic acid regardless of the presence of calcium compounds in the culture medium (Takao, 1965). Schmidt et al. (1981) have stated that oxalic acid produced by brown-rot fungi has a catalytic role in the reduction of ferric iron available in native wood for Fenton-type reactions (Schmidt et al., 1981). Oxalic acid may thus be a key metabolite responsible for cellulose degradation (Akamatsu et al., 1991). Secretion of oxalic acid has been explained as enabling the fungi to weaken the wood structure by increasing the pore size to permit the penetration of lignocellulolytic enzymes (Dutton et al., 1993a). It has been shown that 1 % oxalic acid can reduce the viscosity of cellulose and

hemicellulose. Cellulose undergoes a random hydrolytic cleavage it treated with low concentration of oxalic acid (Shimada et al., 1991). The low molecular weight acids may be important in the initiation of the brown-rot decay by acting on hemicellulose, and ultimately on amorphous cellulose, through the hydronium ion formed from oxalic acid (Green et al., 1991).

The results obtained in this study suggest that the production of hydrogen peroxide is closely connected with the production of oxalic acid in brown-rot decay. The simultaneous appearance of hydrogen peroxide and oxalic acid may result from the action of an extracellular enzyme which generates both hydrogen peroxide and oxalic acid or successive reactions of more than one enzyme coupled to each other. The degradative mechanisms of lignin by white-rot fungi is largely peroxidative; thus these fungi have enzymes that generate hydrogen peroxide. Glucose-1-oxidase and glucose-2-oxidase have been purified from mycelial extracts of *Phanerochaete chrysosporium* and they have been suggested as the major source of hydrogen peroxide (Kelley & Reddy, 1986, Eriksson et al., 1986). Extracellular glyoxal oxidase has been characterized from *P. chrysosporium*. This enzyme activity is induced under secondary metabolism, and its most favourable substrates, glyoxal and methylglyoxal, are produced by the fungus as secondary metabolites (Kersten & Kirk, 1987). Enzymes which oxidize aromatic alcohols to aldehydes, and thus create hydrogen peroxide, are extracellular aryl alcohol oxidases characterized from Pleurotus eryngii and Bjerkandera adusta (Guillem et al., 1990, Muheim et al., 1990). Furthermore, a veratryl alcohol oxidase has been purified from Pleurotus sajor-caju (Bourbonnais & Paice, 1988). No oxidative enzyme system capable of catalyzing the production of extracellular hydrogen peroxide in brown-rot fungi has not been identified despite sustained research.

3.2 THE ROLE OF HYDROLYTIC ENZYMES IN BROWN-ROT DECAY

3.2.1 The cellulolytic system of brown-rot fungi

3.2.1.1 The production of extracellular enzymes by *G. trabeum*

In the present study, the enzymatic hydrolysis of wood polysaccharides was detected by following the production of cellulases and hemicellulases on culture media containing spruce sawdust or microcrystalline cellulose (Avicel) as the sole carbon source by brown-rot fungus *Gloeophyllum trabeum* (II). The preliminary hypothesis was that the hydrolysis of the easily soluble parts of hemicellulose and cellulose takes place by enzymatic reactions. The production of endo-β-1,4-glucanase, which hydrolyses amorphous and substituted celluloses, and endo-β-1,4-xylanase, which hydrolyses the xylan part of hemicellulose, were most

pronounced on both media troughout cultivation. The production and secretion of endoglucanase and xylanase started almost immediately after the inoculation and reached the maximum in 5 - 6 weeks, followed by a sharp decrease, under the cultivation conditions used (Table 3, II/ Fig. 1 and 2). The production pattern of these enzymes was also reflected in the extracellular protein concentration (Table 3, II/Fig. 3). Only trace amounts of β-glucosidase were produced throughout cultivation, without any peak value. The β-glucosidase produced by *Poria placenta* has been observed to be attached to the fungal mycelia (Micales, 1990).

Table 3. The activities of extracellular enzymes and proteins by G. trabeum on sawdust medium after 6 weeks cultivation.

Enzyme	Enzyme activity (nkat/ml)
Endo-β-1,4-glucanase	1.77
Endo-β-1,4-xylanase	1.17
Endo-β-1,4-mannanase	0.03
β-D-xylosidase	0.17
β-glucosidase	0.007
Filterpaper activity *	0.011
Protein concentration **	7.15

* (IU/ml)
** (μg/ml)

The enzyme activities and extracellular protein concentrations observed in cultivations with microcrystalline cellulose as carbon source were lower than one tenth of those obtained on the sawdust medium (Table 4). By following the growth visually in a light microscope, a clear development of mycelial mat was observed. Thus, the fungus was able to grow on crystalline cellulose as the sole carbon source in spite of the low enzyme activity. Contradictory to previous suggestions, (Highley, 1977, Enoki et al., 1991) it was clearly shown in this study that brown-rot fungi can grow on crystalline cellulose without any easily metabolizable carbohydrate, such as glucose or cellobiose.

Table 4. The extracellular carbohydrate-degrading enzyme activites and protein concentration produced by G. trabeum on microcrystalline cellulose after 8 weeks' cultivation.

Endo-glucanase (nkat/ml)	Endo-xylanase (nkat/ml)	Endo-mannanase (nkat/ml)	β-D-xylosidase (nkat/ml)	β-D-glucosidase (nkat/ml)	Filter paper activity (IU/ml)	Protein conc. μg/ml
0.07	0.05	0.06	nd	nd	0.001	0.36

The ability of *G. trabeum* to degrade crystalline cellulose enzymatically was studied by the filter paper method (IUPAC, 1987, Nevalainen & Penttilä, 1995) which measures total cellulase activity. Theoretically, the enzyme system needed for the degradation of filter paper consists of both endoglucanase activity and cellobiohydrolase activity, as well as of β-glucosidase activty (Lundborg, 1989a). The low filter paper activity observed both on the wood based and Avicel media further confirms that *G. trabeum* lacks the ability to degrade the crystalline part of cellulose enzymatically (Table 3 and 4). The trace activities found in the filter paper assay might reflect the release of reducing sugars from the amorphous part of the substrate.

Carbohydrate-degrading enzymes are typically produced by brown-rot fungi in very low quantities and attemps to increase the production of these enzymes by varying the environmental conditions have only met limited success. The formation of these enzymes appears to be largely constitutive (Highley, 1976, Green et al., 1989, Micales, 1990) although none of the cellulolytic genes have yet been identificated by molecular biological methods. In this study the induction of endoglucanase and xylanase from *G. trabeum* took place at the very beginning of the cultivation, which might indicate that the easily solubilized carbohydrate fractions or the easily degradable parts of wood polysaccharides are used at the early stages of growth. It has also been noticed that the hydrolysis of amorphous cellulose or crystalline cellulose, which has been degraded to some extent, can take place by the action of endoglucanases without presence of exoglucanases (Highley, 1988, Highley et al., 1983b). TEM studies on wood treated with endoglucanase produced by *Postia placenta* have shown that the enzyme alone can sufficiently degrade the glycosidic bonds to weaken the microfibril surface and to reduce the strength of cellulose (Murmanis et al., 1988, Lundborg, 1989a). The action of endoglucanase may produce visible changes, but the enzyme alone is very slow compared with the synergistic action of both endo- and exoglucanases. Thus, the enzymatic hydrolysis of cellulose by brown-rot fungi cannot alone explain the rapidly declining strength properties of wood at the initial stage of decay.

The role of the carbohydrate-degrading enzymes in the brown-rot is still questionable. The hydrolysis of crystalline cellulose is believed to be mainly an oxidative, non-enzymatic process, since the cellobiohydrolase activity is absent. The hydrolytic enzymes are too large to be able to penetrate the cell wall structures of intact wood material (Murmanis et al., 1988, Floyrnoy et al., 1989). It has been observed from the microscopic studies that only molecules smaller than 20 kD are able to use the natural pores existing in wood cell walls (Flournoy et al., 1989, Srebotnik & Messner, 1991). An interesting findning is that in brown-rot, the size of natural pores does not change during the decay process as might be expected (Srebotnik & Messner, 1991).

The results obtained in this study clearly show that the hydrolytic activity needed for the degradation of hemicellulose and amorphous cellulose is

produced by *G. trabeum*. However, in future it still needs to be clarified weather the main function of the enzymes is in splitting the oligosaccharides released by the action of the oxidative degradation of wood carbohydrates or to act as the initial tools in the degradation of wood components in order to produce substrates or precursors for the oxidative pathway.

3.2.1.2 The induction and production of endo-β-1,4-glucanase by *P. placenta*

In this investigation the endoglucanase activities produced by *P. placenta* were low both on amorphous and crystalline cellulose (16 and 21 nkat ml-1 at the maximum), whereas on glucose medium the endoglucanase activity increased steadily reaching a maximum (74 nkat ml $^{-1}$) in about 3 weeks (Table. 5). The high endoglucanase activity produced by *P. placenta* on the glucose medium in this study disagree with previous results obtained by Schmidhalter and Canevascini (1992), who observed only β-glucosidase production on glucose. In other investigations, brown-rot fungi have been shown to produce endoglucanases, which appear to be nonresponsive to catabolic repression (Highley, 1973, Highley, 1977, Cotoras & Agosin, 1992).

Each culture medium contained 1 % cellobiose as starter sugar for the growth of fungal mycelia. The growth of the organism was monitored by measuring the mycelial protein content and indirectly, by analysing the changes in the amounts of mono- and oligomeric sugars in the cultures during the growth (IV/Fig. 5 and Table 1). The highest mycelial protein content was obtained on amorphous cellulose medium, on which the growth rate was also highest (IV/Fig. 5). The mycelial protein content was very low on glucose medium, in spite of the high endoglucanase activity. On all culture media, the amount of cellobiose decreased during the cultivation. The uptake of cellobiose was fastest on amorphous cellulose, which correlates with the growth rate and increase in mycelial protein content. Glucose accumulated in the culture liquid on all media, indicating that it is not a preferable carbon source for *P. placenta* (IV/Table 1).

In this study only low endoglucanase activity was detected on crystalline and amorphous cellulose, but activity increased after the accumulation of hydrogen peroxide and oxalic acid ceased. This might suggests that the cellulases and oxidative reactions act synergistically in the degradation of wood carbohydrates or, that the presence of hydrogen peroxide and oxalic acid in the culture media might inactivate the endoglucanase. Higham et al. (1994) noticed the formation of cellobionolactones by the action of cellobiose dehydrogenase in the oxidative degradation of cellulose. Lactones, including cellobionolactone, act as inhibitors of cellulases and β-glucosidase (Higham, 1994). The regulation mechanism for the hydrolytic degradation pathway is still, however, unknown. The simple sugars have been observed to accumulate in the culture medium of brown-rot, and the degradation products are released faster than they are utilized by brown-rot fungi. The enzymes are not repressed by glucose and the decay process

continues regardless of the accumulated oligosaccharides (Lundborg, 1989a, Micales & Highley, 1988, Micales et al., 1989, Murmanis et al., 1987).

Table 5. The production of endoglucanase by P. placenta on 1 % crystalline cellulose, 1 % amorphous cellulose and 0.5 % glucose. Each culture medium contained 1 % cellobiose as additional carbon source.

Cultivation time (days)	1 % Avicel EG activity (nkat/ml)	1 % Walseth EG activity (nkat/ml)	0.5 % Glucose EG activity (nkat/ml)
0	0	0	0
7	3	12	14
14	8	3	44
22	7	7	72
28	21	16	74

3.2.2 The hemicellulolytic system of brown-rot fungi

3.2.2.1 The production, purification and characterization of β-1,4-xylanase of *G. trabeum.*

The degradation of hemicellulose is believed to be an important initial reaction taking place in the brown-rot decay (Micales et al., 1987, Green et al., 1989). Brown-rot fungi have been observed to utilized hemicelluloses prior to cellulose in wood (Highley, 1976, Keilich et al., 1970). Knowledge of the structural and functional properties of the enzymes responsible for the hydrolysis of hemicellulose is therefore extremely important for the development of new strategies in wood protection.

In this study, the brown-rot fungus *G. trabeum* produced the hemicellulose degrading enzymes, of which was the xylanase was most abundant. Only trace amounts of β-mannanase and β-xylosidase activities were detected on sawdust medium. The activity of xylanase in the culture liquid was 18.5 nkat/ml after 9 days of cultivation. The purification of *G. trabeum* xylanase was carried out in two steps. Purification began with a DEAE-Sepharose anion-exhanger at pH 6.5. The activity yield of the first step was 83 %. The pooled xylanase fractions were concentrated and applied to a chromatofocusing PBE 94 column. The second step in purification was gel filtration in a Sephacryl S-100 column. After chromatofocusing and gel filtration the activity yield was 44 % and the purification factor was 15 (III/Table 1). The xylanase appeared as a single band in SDS-PAGE with a molecular mass of 39 kDa. In gel filtration the molecular mass of the xylanase was 42 kDa. The pI of this xylanase was 5.0 (Table 6).

Table 6. Physical properties of purified xylanase of G. trabeum.

Property	Value
Isoelectric point pI	5.0
Molecular mass (kDa)	
SDS-PAGE	39
Gel filtration	42
Temperature optimum (^{o}C)	80
pH optimum	4.0

To date only few enzymes have been purified and characterized from brown-rot fungi (Ishihara et al. 1978, Highley & Wolter, 1987, Green et al., 1989). In this study only one xylanase produced by *G. trabeum* could be detected from the wood based culture medium. The molecular mass of this protein (39 - 42 kDa) is comparable with the xylanases of other basidiomycetes, e.g. brown-rot fungi *Postia placenta* (43 - 45 kDa) and *Tyromyces palustris* (56 kDa) and white-rot fungi *Lentinula edodes* (41 kDa), *Schizophyllum radiatum* (25.7 kDa) and *Schizophyllum commune* (33 kDa) (Ishihara et al., 1978, Green et al., 1989, Mishra et al., 1990, Cavazzoni et al., 1989, Paice et al., 1978). The isoelectric point of *G. trabeum* xylanase was 5.0. The pI values of the previously purified and characterized xylanases from brown-rot fungi were 3.6 in *T. palustris* and 3.8 in *P. placenta* (Ishihara et al., 1978, Green et al., 1989).

The xylanase of *G. trabeum* preferred the most substituted, acetylglucuronoxylan (DMSO-xylan) as substrate, which could be due to the high solubility of the substrate. In *T. reesei* the xylanases have also been observed to prefer the substituted xylans as substrate (Tenkanen et al., 1992). The acetylglucuronoxylan can be regarded as a natural xylan, and 70 % of the original substrate was hydrolyzed within 24 h. The hydrolysis yields of the deacetylated methyl-glucuronoxylan (Roth) and the unsubstituted xylan (Lenzing) were 37 % and 49 %, respectively, when measured as reducing sugars (III/Fig. 3). The xylanase of *G. trabeum* proved to be an endo-hydrolase that produce a mixture of xylooligosaccharides, predominantly xylobiose and xylotriose, as end products (III/Fig. 4). The xylanase of *G. trabeum* did not show notable endo-β-1,4-glucanase, endo-β-1,4-mannanase or β-xylosidase activity. The xylanase exhibited β-glucanase activity with barley β-glucan as substrate (III/Table 3).

The extracellular carbohydrate-degrading enzymes from the brown-rot fungi have been reported to be relatively stable (Highley & Wolter, 1987). The xylanase of *G. trabeum* had its pH optimum at pH 4, and it was found to be have a very high temperature optimum. In natural conditions in Finland *G. trabeum* is known to grow vigorously at enhanced temperatures, e.g. in saunas and flat roof constructions. The optimal temperature of the enzyme was 80 °C in a 30 min incubation. After 2 h incubation at 70 °C 22 % and after 24 h at 60 °C about 50 % of the activity remained (III/Fig. 2). The optimal temperatures of the xylanases of

P. placenta and *T. palustris* were 50 - 60 °C and 76 °C for 24 h and for 30 min, respectively (Green et al., 1989, Ishihara et al., 1978). However, the xylanase of *G. trabeum* seemed to have higher temperature optimum than these two brown-rot xylanases. The thermostability of the xylanase of *P. placenta* decreased sharply at 60 °C after 1 h incubation (Green et al., 1989). According to Ishihara et al. (1978) the xylanase of *T. palustris* was stable at 70 °C for 30 min. Compared to xylanases purified from soft-rot fungi, e.g., *Trichoderma sp*. (Viikari et al., 1993, Yu et al., 1987), the *G. trabeum* xylanase is remarkably thermostable. The optimal temperatures of most hydrolytic enzymes produced by basidiomycetes are around 50 °C, and the enzyme activities are noticed to decline quickly at higher temperatures (Comtat, 1983, Hoebler & Brillouet, 1984, Ishihara et al., 1978, Paice et al., 1978, Green et al., 1989, Mishra et al., 1990, Cavazzoni et al., 1989).

The brown-rot polysaccharide-degrading enzymes are quite tolerant to inhibitors (Green et al., 1989, Highley & Micales, 1990). In this work, the inhibitory effect of various metal chlorides, chelators and wood extractives on the xylanase activity produced by *G. trabeum* was studied. The aluminium chloride was the only compound found to have an inhibitory effect on the xylanase activity. The residual xylanase activity was 77 % after 30 min incubation in 10 mM aluminium chloride. This result is similar to those obtained with xylanases of *Trichoderma reesei* and *Bacillus circulans* (Buchert et al., 1993a). Other metal chlorides, metal chelators (EDTA, STPP) and wood extractives did not have any inhibitory effect on the xylanase. (III/Table 4). The xylanase of *P. placenta* has been reported to be a glycoprotein (Green et al., 1989). The carbohydrate content of the xylanase of *G. trabeum* was not studied in the present context, but it is reasonable to believe that the structures of the brown-rot enzymes are quite similar. The strong inhibitory effects may be associated with the carbohydrate moiety of the enzyme (Highley & Micales, 1990).

Xylanase is one of the main hydrolytic polysaccharide degrading activities in brown-rot fungi (Table 3) (Highley et al., 1983a, Messner et al., 1984). In a softwood cell wall, most of the glucuronoxylans are located in the S1-layer (Sjöström, 1981). Therefore it is possible that the utilization of the arabino-4-methyl glucuronoxylans is the initial event in the degradation of hemicellulose by brown-rotters, regardless of the fact that softwood hemicellulose is composed mainly by galactoglucomannans. The low mannanase activity produced by *G. trabeum* could be explained by this, or it could be due to the substrate specificity, for this enzyme might not have the same activity on artificial substrates, such as sawdust, as on solid wood (II).

The removal of hemicellulose is believed to be one of the main early events in brown-rot decay. It is therefore important to understand the role of xylanases as a part of the decaying system of brown-rot. Furthermore, thermophilic xylanase has interesting properties with respect to industrial applications. The use of thermophilic xylanases in bleaching of cellulose pulps in order to minimize the use of environmentally harmful chlorine

compounds is a possible application for fungal hydrolytic enzymes (Viikari et al., 1986, Viikari et al., 1987, Kantelinen et al., 1988).

3.3 THE ROLE OF FENTON-TYPE REACTION IN WOOD CARBOHYDRATE DEGRADATION

A biomimetic approach, consisting of components mimicking biological processes, was used to clarify the role and importance of the Fenton-type reaction in the carbohydrate degradation by brown-rot fungi.

Spruce sawdust and microcrystalline cellulose (Avicel) were modified in the H_2O_2/Fe(II) treatment as indicated by the weight loss of the substrates (Table 7). Reducing sugars were not significantly accumulated and no solubilized mono- or oligosaccharides could be detected by the HPLC (data not shown). The identification of the degradation products released in the Fenton treatment remains to be studied.

Table 7. The effect of Fenton reaction (H_2O_2/Fe^{2+}) on spruce sawdust and microcrystalline cellulose (Avicel).

Substrate	Hydrogen peroxide conc. (%)	Weight loss (%) of original d.w.	Reducing sugars (%) of original d.w.
Spruce sawdust	0	1.3	0.8
	0.2	9.6	0.9
	1.0	23.7	0.6
Avicel	0	0.8	0.4
	0.2	2.5	1.8
	1.0	9.2	1.2

The degree of hydrolysis of the pretreated spruce sawdust was clearly increased when treated with the complete cellulase preparation, Econase CE, obtained from *T. reesei*. The increased accessibility of the pretreated spruce sawdust for enzymatic hydrolysis with purified EG I and *P. placenta* endoglucanase was even more evident (Table 8). Analysis of the Fenton treated sawdust indicates that H_2O_2/Fe^{2+} primally attacks the hemicelluloses in wood, thus increasing the accessibility of the cellulose. The relative content of hemicelluloses had decreased significantly. With 1 % H_2O_2 loss of galactoglucomannan and xylan represented two thirds of total weight loss (Table 9).

Table 8. The enzymatic hydrolysis of Fenton treated (1 % H_2O_2/Fe^{2+}) spruce sawdust with Econase CE, T. reesei EG I and P. placenta endoglucanase. For the endoglucanase production, P. placenta was cultivated on glucose (0.5 %) and cellobiose (1 %) containing medium. The weight losses obtained on Fenton treated samples are counted from 23.7 % degraded substrates.

Treatment	Enzyme	Weight loss (%) of original d.w.	Reducing sugars (%) of original d.w.
Reference enzymatic hydrolysis	Econase CE	15.6	17.1
	EG I	5.4	3.6
	P. placenta EG	1.6	1.1
Fenton treatment and enzymatic hydrolysis	Econase CE	32.8	32.5
	EG I	14.9	10.0
	P. placenta EG	4	3.8

Table 9. Chemical composition of H_2O_2/Fe^{2+} treated spruce sawdust. The amounts of the structural compoundsare expressed as grams of the dry weight yield of the sample.

Concentration of H_2O_2 (%)	Dry weight yield of the sample (g)	Lignin (g)	Xylan (g)	Mannan (g)	Cellulose (g)	Araban (g)	Galactan (g)
0	10.8	2.9	0.9	1.9	4.7	0.04	0.09
0.2	9.9	3.0	0.7	1.3	4.7	0.01	0.06
1.0	7.9	2.5	0.5	0.6	4.2	0.01	0.04

The pretreatment with H_2O_2/Fe^{2+} decreased the degree of hydrolysis of microcrystalline cellulose treated with a complete cellulase preparation. The oxidative pretreatment of pure cellulose apparently has a modifying effect on the substrate, which in turn prevents the action of cellobiohydrolase of the complete cellulase preparation. The oxidative treatment can be expected to produce oxidated groups at the chain ends of cellulose which cannot be attacked by cellobiohydrolases, thus reducing total hydrolysis. Although the H_2O_2/Fe^{2+} pretreatment decreased the hydrolyzability of pure cellulose when treated with the complete cellulase preparation, the hydrolyzability with both purified EG I of *T. reesei* and endoglucanase obtained from *P. placenta* was increased (Table 10). This result clearly demonstrates that an oxidative treatment enhances enzymatic action when no cellobiohydrolase activity is present. Neither pure endoglucanase from *T. reesei*, nor the endoglucanase of *P. placenta* were able to degrade pure cellulosic substrates or fine ground wood (Table 8 and 10). After oxidative treatment with Fenton´s reagent, however, the hydrolysis of both pure cellulose and wood was substantially increased by these enzymes.

Table 10. The enzymatic hydrolysis of Fenton treated (1 % H_2O_2/Fe^{2+}) microcrystalline cellulose with Econase CE, T. reesei EG I and P. placenta endoglucanase. For the endoglucanase production, P. placenta was cultivated on glucose (0.5 %) and cellobiose (1 %) containing medium. The weight losses obtained on Fenton treated samples are counted from already 9.2 % degraded substrates.

Treatment	Enzyme	Weight loss (%) of original d.w.	Reducing sugars (%) of original d.w.
Reference enzymatic hydrolysis	Econase CE	58.8	57.5
	EG I	6.3	5.8
	P. placenta EG	0.3	1.4
Fenton treatment and enzymatic hydrolysis	Econase CE	50.1	49.6
	EG I	9.0	7.7
	P. placenta EG	4.3	3.3

H_2O_2 and Fe(II) have been shown to decompose cellulose. At the primary stages of decomposition, the substrate disintegrates into very short fibres, which are subsequently solubilized into non-accumulating organic matter (Halliwell, 1965). The high reducing capacity of brown-rotted cellulose indicates extensive oxidation of cellulose coupled with weight loss via hydrolysis (Koenigs, 1972a, 1972b, Highley, 1977). According to Kirk et al. (1991) brown-rotted cellulose resembles cellulosic substrates treated with H_2O_2/Fe^{2+}. Hydroxyl radicals are formed in the Fenton reaction. Illman et al. (1988a, 1988b) and Backa et al. (1992) have shown that hydroxyl radicals are formed at the initial stage of brown rot decay. It has been observed that highly reactive OH radicals react faster with lignin-like aromatic compounds, but as unselective species they also attack the carbohydrate containing compounds, such as cellulose and starch, bringing about advanced breakdown (Moody, 1964, Ek et al., 1989, Walker et al., 1995). In this study, it was observed that the amount of lignin in Fenton treated spruce sawdust decreased slightly (Table 9).

In this study, it was clearly observed that the pretreatment of wood substrate with H_2O_2/Fe^{2+} enhances its susceptibility to enzymatic saccharification. Takagi (1987) obtained similar results by treating of the lignocellulosic materials with hydrogen peroxide and manganese. The result indicated that the H_2O_2/Mn-system brings about neither removal of lignin nor a decrease in the crystallinity of cellulose, but renders the linkage of cellulose, hemicellulose and lignin loose, resulting in a higher suscebtibility towards enzymatic hydrolysis (Takagi, 1987). It is quite obvious that Fenton-type oxidative reactions play a crucial role in enhancing the initial stage of brown-rot decay by breaking the bonds in the cell wall components, modifying the wood substrate and thus making it more accessible for the hydrolytic degradation pathway.

4 CONCLUSIONS AND FUTURE PROSPECTS

At the initial stage of wood polysaccharide degradation, the brown-rot fungi seem to operate with a mechanism which causes extensive changes in the wood cell wall structure, leading to a rapid decline in of its strength properties. Brown-rot fungi have been presumed to produce a low molecular degradation agent which is capable of penetrating into the wood cell wall structures. In the present study, it was observed that the production of extracellular hydrogen peroxide was most pronounced on cellulosic substrates, whereas on glucose only low amounts of hydrogen peroxide were detected. Interestingly, the production of hydrogen peroxide was closely connected with the production of oxalic acid, which brings about the acidic growth conditions typical for brown rot. The production patterns of the two compounds were very similar. This simultaneous appearance of hydrogen peroxide may result from the action of an extracellular enzyme which generates both hydrogen peroxide and oxalic acid or successive reactions of more than one enzyme, possibly coupled to each other. In the first stage of this work, it was observed that the production of hydrogen peroxide by brown-rot fungi was induced by the degree of cellulose crystallinity. This discovery suggests that crystalline structure or highly ordered chemical conformation of the substrate act as inducer for the enzyme(s) catalyzing the production of extracellular hydrogen peroxide by brown-rot fungi. In later quantitative experiments it was further demonstrated that cellulosic substrates were the primary inducers of the hydrogen peroxide production by *P. placenta.*

The present investigation clearly shows that brown-rot fungi produce extracellular hydrogen peroxide which may be able to penetrate into the wood cell wall structures and to react with the endogenous iron or other transition metals producing reactive radicals through Fenton type of reaction. These chemical reactions in the wood cell wall act as a pretreatment leading to the modification of the woody substrate. In this study it was observed that due to the Fenton treatment, especially the hemicellulosic components of the spruce sawdust were cleaved, which in turn increased the accessibility of the cell wall structure for the hydrolytic enzymes. Thus, it is probable that in addition to acting as a cellulose modifying tool, the Fenton reaction also provides a more porous cell wall structure, enabling the penetration and action of hydrolytic enzymes.

The role of cellulolytic enzymes in brown-rot decay is still quite unclear. Brown-rot fungi lack detectable cellobiohydrolase activity needed for the degradation of crystalline cellulose. Brown-rot fungi, however, produce endoglucanases, although the amount of these enzymes is quite low compared with the enzyme activites obtained from other cellulolytic microorganisms, e.g. *T. reesei*. The production of endoglucanases by brown-rot fungi was found to be constitutive and not to be repressed by glucose. Regardless of the low amount of cellulases produced by these fungi, it is likely that after the pretreatment of wood polysaccharides by hydrogen peroxide mediated reactions, the hydrolytic enzymes can penetrate

to the cell wall and act vigorously on the modified cellulose fibrils. This leads to the formation of smaller oligosaccharides which in turn are used in the fungal metabolism. The hypothetical degradation mechanism of brown-rot decay suggested by the results obtained in this and previous studies is presented in Fig. 8.

Current used wood preservation methods are based on the general broad spectrum toxicity of preservative compounds. The impact of wood preservatives is directed towards the basic metabolic reactions common to all living organisms. Due to their non-specificity, serious health (e.g. carcinogenicity) and environmental (soil and water contamination) risks are involved with conventional wood preservation. An understanding of the physiological and biochemical basis of the decomposition mechanisms of the wood decay fungi is extremely important for the development of new, environmentally safe wood preservation methods. Since the fungal wood degradation system consists of fairly unique reactions, the new prevention methods can be focused specifically on key points along the degradation pathway. The information obtained in this study suggests that prevention of the oxidative degradation of cellulose offers an excellent target for the new specific wood preservation methods, taylor-made against fungal degradation reactions. Further research will be focused on the induction and inhibition of the enzymatic system catalyzing the production of extracellular hydrogen peroxide. Nutritional demands, especially the nitrogen metabolism of decay fungi, also provide an interesting target for novel wood preservation systems. The methods based on biological antagonism, e.g. the use of microbial siderophores, and the methods based on the natural durability of trees, could also provide feasible alternatives for modern wood protection.

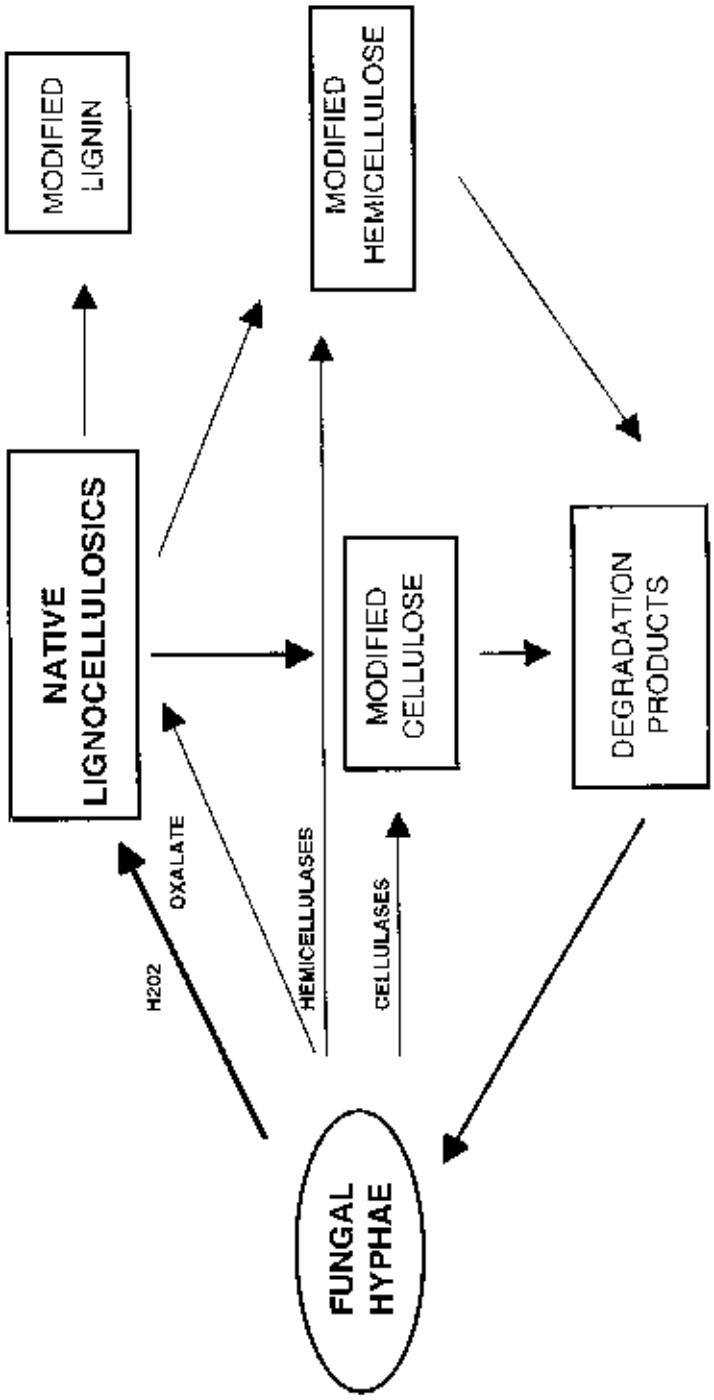

Fig. 6. The hypothetical degradation mechanims of brown-rot fungi in the light of the results obtained in this study and previous works.

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