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"Wood biotechnology - the utilization of fungal decay mechanisms"

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Wood biotechnology - the utilization of fungal decay mechanisms

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Introduction

Whenever biomass is considered for utilization, wood is the dominant material. It is the greatest source of biomass on earth. Besides trees, shrubs and vines are also classified as woody plants. However, especially softwood trees with straight stems and slight taper are suited best for mass production. In industrialized countries there are competing uses for wood supply. In addition to its use for construction and masonry as well as for energy production, wood is an important chemical raw material, especially for fiber production.

Great effort was put into the detection of the enzyme mechanisms involved in the decay of wood by microorganisms in the past. As a result, the breakdown of cellulose and hemicellulose can be regarded as largely understood. Lignin breakdown, however, is still on its way to be discovered. Through the fast growing knowledge of microbial breakdown mechanisms and the increasing interest in environmentally friendly technologies, a new scientific branch is evolving which could be termed „wood biotechnology“. The objective is to apply either living microorganisms or their isolated enzymes for the conversion of lignocellulosic materials. Presumably, most of these biotechnologies will be integrated into chemical processes.

The objective of this paper is to emphasize the biological, ultrastructural and biochemical background of microbial decay mechanisms of wood and to demonstrate the prospective potential of wood biotechnolgy.

Colonization of wood by fungi

When a fungus degrading a log or the trunk of a living tree is recognized, mostly only its reproductive structure, the fruit body is seen. The major part of its biomass is hidden in the depth of the wood. Microscopically thin branched threads of a diameter of appr. 0.01 mm - the fungal hyphae - grow through the lumina of the wood fibers, degrade the wood cell walls by excreted enzymes and transport the metabolites to the outside of the wood to produce fruit bodies.

When wood is used as a raw material to produce chemicals it is debarked and chipped. The main port of entrance and shortest way into the interior of debarked wood are the rays. They lead radially from the surface of a debarked log, or a wood chip, directly into the center. Easily available carbohydrates and nitrogen are provided for fungal growth by the parenchyma cells. From the rays, the fungal hyphae spread into the adjacent tracheids. Early wood is colonized first, with hyphae passing from cell to cell via the pit apertures or by bore holes, formed by extracellular enzymes, through the wood cell walls. When the easily degradable compounds are consumed - they amount to appr. 3% of the wood substance in pine or spruce, but may be as high as almost 10% - the wood cell walls are attacked by the extracellular enzyme system. Depending on the composition of these enzymes either the carbohydrate or the lignin fraction may be degraded preferably, leading to characteristic decay types.

Patterns of microbial breakdown of wood:

Fungi are the main decomposers of wood in nature. Taxonomically, two groups involved in wood decay, can be distinguished:

- **Ascomycotina and related Deuteromycotina**, also called *molds*
- **Basidiomycotina,** partially known as *mushrooms*

As far as is known today, bacteria play a comparably minor role in wood decay.

Wood decay is a very complex process interlinking ezyme systems for cellulose- hemicellulose- and lignin-degradation. The mechanisms involved vary between fungal groups. Depending also on wood species and partially on physiological parameters, different types of decay can be distinguished morphologically. The main wood decay types are:

Blue stain fungi and molds:

They can hardly be considered to be decay fungi. They are able to colonize wood without degrading structural components of wood. They consume only the easily available wood components like extractives (fatty acids, resin acids, triglycerides sterol esters etc.) or non-structurally bound carbohydrates. The most abundant fungi of this type are species of *Trichoderma*. Some fungi also excrete dark pigments leading to a dark stain of the wood, termed blue stain fungi. Abundant species are *Aureobasidium pullulans, Sclerophoma pityophila, Ophiostoma spp.* Dark pigments may also be produced by soft rot fungi.

Soft rot: Ascomycotina and Deuteromycotina.
Similar decay patterns are produced by bacteria.

Morphology:

The term soft rot refer to the soft appearance of the wood surface after decay. Dried wood has cracks and checks and is of brown color, similar to brown rot. Chains of biconical and cylindrical cavities are produced within the secondary wall. Additionally, the wood cell walls may be eroded from the lumen surface. Similar to brown rot, loss of carbohydrates predominates lignin loss during decay. The biochemical mechanism of decay is not well understood.

Fungal species:

Phialophora hoffmannii, Ph. fastigiata, Humicola alopallonella, Dactylomyces crustaceous, Thielavia terrestris, Lecytophora sp., Penicilium spp., Chaetomium globosum, etc.

Brown rot: Basidiomycetes.

Morphology:

Extensive degradation of cellulose and hemicellulose but only chemical modification and slight degradation of lignin leads to a dark brown crumbly residue mainly consisting of lignin. The main feature is the rapid depolymerization of hemicellulose and the amorphous part of cellulose already in the early stages of decay, resulting in a dramatic decrease in wood strength. The *dry rot* fungus *Serpula lacrimans*, detrimental to wood in construction belongs to this group. Brown rot predominates on soft wood.

Fungal species:

Coniophora puteana, Gloeophyllum abietinum, G. sepiarium, Paxillus panuoides, Poria placenta, Serpula lacrimans, S. himantioides, Lentinus lepideus, L. cyathiformis, Piptoporus betulinus, Fomitopsis pinicola, Tyromyces spp.

White rot: Basidiomycetes.

Morphology:

It appears in a broad range of morphological patterns. The common features are the bleached white colour of advanced decay and the ability to degrade lignin extensively. Based on the decay rate of lignin to carbohydrates, two main patterns can be distinguished. Some fungi belong to either of the patterns, depending on the type of wood. White rot predominates on hard wood.

Simultaneous white rot:

The decay starts at the lumen surface and progresses continuously towards the middle lamella resulting in a thinning of the cell walls. In late stages of decay only cell corners might remain. Cellulose, hemicellulose and lignin are degraded more or less at the same rate.

Fungal species:

Polyporus (Coriolus) versicolor, Trametes hirsuta, T. gibbosa, Schizophyllum commune, Fomes fomentarius etc.

Selective white rot:

Lignin and hemicellulose is degraded predominantly to cellulose. Degradation of lignin starts at the lumen surface and progresses down to the middle lamella. The lignin degrading system also depolymerizes the middle lamellae and cell corners. As cellulose remains unattacked for a long time, the diameters of the cell walls remain approximately the same. Due to the dissolution of the middle lamellae, the fibers are separated from each other. Because of the strong and selectively acting ligninolytic system, these fungi have a great potential for biotechnological purposes.

Fungal species:

Ceriporiopsis subvermispora, Phlebia tremellosa, Dichomitus squalens, Ganoderma applanatum, Phanerochaete chrysosporium (on hard wood)

Enzymes involved in the degradation of plant cell walls:

Lignin depolymerizing oxidative enzymes and their accessory enzymes:

The enzymes giving wood degrading fungi their unique ability to degrade highly lignified cell walls are the ligninolytic enzymes. Their reaction mechanism is an oxidative cleavage reaction. At the time being we distinguish three main lignin degrading enzymes:

Lignin peroxidase (LiP) and manganese peroxidase (MnP):

Both are oxidized by hydrogen peroxide which is produced by accessory enzymes. LiP and MnP are heme-containing peroxidases, which catalyze one-electron oxidations of various aromatic substrates.

The primary reducing substrate for LiP can be phenolic or non phenolic lignin, resulting in the production of aryl cation radicals further undergoing various non-enzymatic reactions with a wide range of degradation products. The catalytic cycle of LiP includes veratryl alcohol as co-substrate. Due to the high redox potential of +1.5 V, it is a unique enzyme capable of oxidizing a broad range of aromatic compounds.

The reducing substrate for MnP is free manganese ion Mn 2+. In the presence of an appropriate chelator a complex is formed which is able to oxidize phenolic substrates via the formation of phenoxy radicals. As outlined in the chapter on the ultrastructure of wood decay, the low molecular weight of this complex enables it to diffuse into the sound wood cell wall, contrary to the enzyme itself. In this way it may act as a mediator, mediating the enzymatic cleavage of lignin over the long distance from the lumen surface of the wood fiber down to the middle lamella.

Laccase (Lac)

Is a copper containing polyphenol oxidase, which is oxidized by molecular oxygen by transferring four electrons. It is reduced by a wide range of phenolic substrates, which are oxidized by one electron oxidation. One possible function of Lac might be the detoxification of low-molecular weight quinones and phenols produced during lignin degradation, but participation of Lac in lignin degradation has also been indicated. It has been shown that also the reaction mechanism of Lac may include a low-molecular weight mediator.

Accessory enzymes:

Extracellular as well as intracellular enzymes are produced by wood decay fungi to provide H2O2 for the action of the peroxidases LiP and MnP:

glyoxal oxidase (GLOX, extracell.)
aryl-alcohol oxidase (AAO, extracell.)
glucose-1-oxidase (GO)
pyranose-2-oxidase (POD)

Other enzymes are involved in the reduction of phenoxy- and cation radicals produced by LiP and MnP and the oxidation of saccharides derived from carbohydrate breakdown, thus combining these two routes of wood cell wall degradation:

cellobiose:quinone 1-oxidoreductase (CBQ)
cellobiose dehydrogenase (CDH)

The breakdown mechanism of lignin, as carried out by the living fungus, is very complex, involving several enzymes in a concerted action we do not fully understand yet. Nowadays our knowledge is rather based on results on lignin model compounds and only recently the first successful application of these enzymes in degrading native lignin in unbleached pulp has been reported. Nevertheless, the prospective potential of ligninolytic enzymes for the utilization of wood and other lignocellulosic substrates is huge.

Polysaccharide cleaving enzymes:

A vast number of fungi is capable of colonizing lignocellulosic substrates in nature, degrading either easily degradable extractives or cellulose and hemicellulose, unprotected by lignin in plant cell walls with a low level of lignification by excreting enzymes to hydrolize the cell wall polysaccharides. The main enzymes involved are:

Cellulases:	endoglucanase exoglucanase cellobiohydrolase
Hemicellulases:	xylanases mannanases pectinases

In this paper the enzyme mechanisms for cellulose and hemicellulose degradation are not dealt with in detail, as these enzymes are also produced by many non-wood degrading fungi. As far as they are applied industrially, they were obtained from fermentations with molds like *Trichoderma reesei.*

An interesting, so far not well understood mechanism of cellulose and hemicellulose depolymerization is applied by brown rot fungi. Electron microscopic results indicate that the agent must be low molecular, diffusing rapidly through the whole wood cell wall. Peptides and siderophor-type agents have been described to be produced by these fungi, catalyzing a Fenton-type reaction where hydroxyl radicals are produced. These are known to rapidly depolymerize amorphous cellulose and hemicellulose which happens in brown rot already at a few per cent weight loss.

Besides the enzymes described above, numerous other enzymes are excreted by wood rotting fungi. Because of their technological relevance only the lipases should be mentioned. These enzymes hydrolize the triglycerides, which constitute a considerable amount of the extractives in soft wood, to fatty acids and glycerol, which in turn are metabolized by the fungus.

Ultrastructure of wood decay:

While the interaction between enzymes and substrate can be reached easily in liquid culture, the ultrastructural architecture of the undecayed wood cell wall largely restricts the penetration of the cell wall layers by enzymes and thus prevents free access to the enzyme substrate. In wood, the cristalline cellulose microfibrils are surrounded by amorphous hemicellulose macromolecules, bound to cellulose mainly by hydrogen bonds. The voids between the carbohydrate aggregates are filled with the amorphous matrix of lignin. The hemicellulose molecules reach into the lignin matrix to which they are chemically bound.

The restricted access of both carbohydrate cleaving hydrolases and the oxidative ligninolytic enzymes to their substrate can be explained theoretically by relating the pore size distribution measured in wood to the size of enzymes excreted by fungi. Adapting the solute exclusion technique to wood, the frequency distribution of capillary dimensions in water-swollen spruce wood was determined to be appr. 10 Å for the median and 35 Å for the maximum dimensions of the capillaries. In comparison, the tadpole-shaped enzyme cellobiohydrolase (CBHI) of *Trichoderma reesei* having a molecular weight of 58 kD was determined to have a maximum diameter of 44 Å and a length of 180 Å . Considering the hydrodynamic diameter of the dissolved solute enzyme, a free diffusion into the wood cell wall can be even excluded for the gross capillaries. Although the shape and size of ligninolytic enzymes has not been determined yet it can be assumed that the same applies to this group of enzymes, taking into account their molecular mass ranging between 38 and 80 kD.

Our knowledge of the localization of ligninolytic enzymes within hyphae of white-rot fungi and wood cell walls was considerably increased when the transmission electron microscopic immuno-cytochemical label technique was adopted to ligninolytic fungi and wood. This method comprises two steps. Firstly, primary antibodies raised against ligninolytic enzymes or other proteins are bound to the ligninolytic enzymes on the surface of an ultrathin section of wood. In a second step commercial gold labeled secondary antibodies are bound to the enzyme-primary antibody complex. By this technique, the proteins to be investigated are localized by the electron dense gold particles, shown in micrographs as black dots.

When uncolonized pine wood and wood degraded by *Phanerochaete chrysosporium* was investigated by the immonogold labeling, neither the uncolonized wood cell walls nor cell walls of advanced stages of decay at 25% weight loss were penetrated by LiP enzymes. In decayed cell walls, penetration was only found in areas of heavy attack where a morphological change of the cell wall had taken place.

The technique was extended by infiltrating wood with marker proteins of a similar molecular weight as that of ligninolytic enzymes (ovalbumin, 45 kD) and a lower molecular weight (myoglobin, 16.5 kD), respectively. With both marker proteins, the results remained essentially the same as with LiP. They were unable to penetrate undecayed wood cell walls. Recently, it was shown by other researchers that wood cell walls colonized for 4 to 8 weeks by the selective white rot fungus *Ceriporiopsis subvermispora*, can be infiltrated partially by the very small marker protein insulin (5.7 kD).

When selectively delignifying fungi like *Ceriporiopsis subvermispora* or *Dichomitus squalens* are cultivated on wood chips for approximately 8 weeks, wood fibers may undergo a total separation by dissolution of the middle lamellae. Most of these fibers only show weak signs of corrosion of the lumen surface and the outer surface. Nevertheless, infiltration of these cell walls with LiP, MnP, myoglobin and ovalbumin, respectively, gave negative results for cell wall penetration. These findings indicated that both initial modification as well as solubilization of lignin are caused by a low molecular mass agent much smaller than ligninolytic enzymes and even smaller than myoglobin. As only fungi excreting ligninolytic enzymes are capable of degrading wood cell walls, these low molecular mass agents must be part of a reaction chain initiated by the ligninolytic enzymes. Various compounds possibly mediating the cleavage reactions in the lignin molecule have been proposed in the literature.

Investigations into the catalytic cycle mediating lignin degradation is one of the main current topics of research on wood decay .

Biotechnological processes

The message to be taken from the ultrastructure of wood decay is that only processes where the living fungi are used make sense if wood is to be modified biotechnologically. The living fungi, growing into the wood chips by their hyphae, have the advantage of being a transport system as well as a production system for the wood modifying enzymes.

As soon as wood is separated into its fibers and delignified by chemical cooking, the porosity of the fibers is drastically increased by the extraction of part of the lignin, enabeling enzymes to penetrate the cell walls.

Thus, two types of technologies can be distinguished: fungal technologies and technologies where isolated enzymes are used. So far, developments in wood biotechnology are more or less restricted to pulp and paper production.

Fungal technologies:

As mentioned above, wood is chipped to pieces of appr. 5 cm length, prior to mechanical or chemical pulping. These chips are piled up to huge heaps, containing the amount of wood needed for 2 - 4 weeks production. The chip piles act as a buffer of raw material and are a good opportunity for fungal processes to be introduced. One new process has reached already the production scale:

Pitch reduction:

Another reason why chip piles are kept is that the indigineous fungal flora developing in the pile degrades resin, known to create stickies on the paper machine when fresh wood is used. The company CLARIANT selected a colorless strain of the fungus *Ophiostoma piliferum* from a chip pile which usually is dark. This strain has an exceptionally high potential to hydrolyze triglycerides and to consume the degradation products. Spores of this fungus are sprayed on wood chips when the pile is set up. After 2 weeks of cultivation, 90 % of esterified fatty acids were degraded, compared to 74% on uninoculated piles and the free fatty acids were reduced by 50% compared to 20%, respectively. Furthermore, the white fungus competes successfully with blue stain fungi, resulting in a higher brightness level after bleaching. The process is being adjusted to various wood types.

Biopulping:

A similar process, but with the application of selectively delignifying white rot fungi, is being developed by the Forest Products Laboratory in Madison, USA and by our lab in Vienna. This group of fungi has the advantage of creating only appr. 2% weight loss, by consuming mainly extractives within the first 2 weeks. As a result of a screening programme *Ceriporiopsis subvermispora* was selected for application. In this process, the wood chips are briefly steamed to kill the competing fungal flora, then they are inoculated with a fungal suspension and a cheap liquid medium. The optimum time for pretreatment is two weeks. After this period the fungus has colonized the chips and excreted the ligninolytic enzymes, leading to a depolymerization of the lignin matrix of the wood cell walls.

When chips are cooked in the sulfite process, the lignin content of the cooked pulp is reduced by 30 - 50% at a kappa level of 24 of untreated chips. This effect can either be used to reduce the cooking time and increase the capacity of the cooker or to end up with a bleached pulp, lower in lignin content. The reduction in fiber strength is very low.

When pretreated chips are refined to produce mechanical pulp, energy savings of 30% was the result. Obviously due to an increase in free cellulose fibrils on the surface, the strength of handsheets increased by 150%. Caused by a browning of wood chips during cultivation, a slight decrease in brightness had to be accepted.

Additionally to the biopulping effect *Ceriporiopsis subvermispora* decreases the content of triglycerides by at least the same extent as this is achieved with *Ophiostoma.*

At present the biopulping process is in the scale-up stage. Experiments will show if a slightly aerated chip pile will be sufficient or if the process will have to be run in a silo.

At the time being, this technology is developed only to pretreat wood for pulping. It can be immagined that it could also be useful as pretreatment step for other technologies for wood utilization.

Biocontrol:

It has been known for quite a long time that some fungi exhibit strong antagonistic activities against other fungi. Especially strains of the mold fungus *Trichoderma harzianum* are able to inhibit the growth of wood degrading basidiomycetes. It was shown that the inhibitory effect is caused by soluble compounds as well as by volatiles. The biocontrol effect is stronger against brown rot fungi than against white rot. In laboratory tests selected strains were able to completely prevent weight loss of wood blocks by brown rot fungi. Electricity poles impregnated with *Trichoderma harzianum* are exposed in field trials to evaluate the performance of biocontrol under natural conditions.

Bioremediation of wood impregnated with creosote:

The disposal of electricity poles and railway sleepers after service life creates environmental problems. Incineration is one of the solutions but to prevent emission of toxic polyaromatic hydrocarbons (PAH), technologically sophisticated incineration plants are needed, making incineration an expensive process. PAH are chemically related to lignin and are substrates for ligninolytic enzymes. Selected white rot fungi show a high threshold for PAH and are able to oxidize these compounds under laboratory conditions, making them potential candidates for fungal remediation of creosoted wood. A more feasible process will possibly be the use of isolated ligninolytic enzymes.

Enzymatic processes:

Delignification with ligninolytic enzymes:

After appr. ten years of worldwide research into the ligninolytic enzyme system, the first successful results of the application of these enzymes for delignification in a commercial bleaching process were reported recently. The process of the German company LIGNOZYM is based on a laccase-mediator system and was tested under pilot conditions. Starting from a 40% ISO brightness level, 50% ISO brightness of a soft wood kraft pulp was reached after laccase treatment and an extraction stage. The lignin content of the pulp was reduced from kappa 10.7 to 6, indicating a strong depolymerizing effect of the laccase-mediator system. For this experiments 2 kg enzyme and 13 kg mediator were used per ton of pulp in the unoptimized process. Much lower amounts have to be expected after optimization.

Application of hydrolases in pulp bleaching and paper making:

Xylanase bleaching:
The first enzymes to be used in the pulp and paper industry were xylanases. When a xylanase step is included in a ECF bleaching sequence on kraft pulp, the chlorine consumption can be reduced by appr. 20%. The underlying hypothesis is that by degrading the reprecipitated xylan the extraction of lignin particles is facilitated. Xylan supported bleaching is used in several paper mills.

Deinking:
Xylanases and cellulases are investigated to be used for deinking processes. By cleaving the surface fibres of recycled pulp, the detachment of ink particles should be facilitated.

Improvement of runnability of paper machines:
When bleached pulp is treated with cellulase for a short time, the microfibrils at the fiber surface are cleaved off. This reduces the capillary forces, resulting in a shorter time needed for dewatering on the paper machine and thus increasing its running speed. This process is of even greater importance when recycled pulp is used, due to the higher content of fines.

Pitch reduction:
Lipase, the enzyme responsible for pitch reduction in the fungal process can also be applied as isolated enzyme on chemical pulp. In a pilot test, 90% of triglycerides were degraded within 3 hours on sulfite pulp.

Conclusions

The biotechnological approaches to using fungi or their isolated enzymes to modify wood or wood components shown above demonstrate the great potential of biotechnological processes for the wood industry. We are only at the beginning of a new technological era. Further research will provide better insight into the reaction mechanisms, will lead to improved production systems for enzymes and certainly will pinpoint new fields of application. Principally wood degrading fungi are capable of carrying out all the reactions needed to mineralize wood.