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BIOTECHNOLOGY RELATED TO WOOD PRESERVATION

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Summary

Biotechnological processes for converting lignocellulosic substrates have been implemented successfully in pulp and paper production. While in many of these applications wood components are primarily depolymerised by enzymes, the main goal in wood preservation is to prevent wood deterioration by microbial enzyme systems. These two objectives are very different from each other. The target of preventing wood degradation by biotechnological processes is more complex, which might be the main reason why at present none of the latter processes has been implemented on industrial scale in the wood preservation industry. Impregnability improvement of refractory wood species, biocontrol, bioremediation of treated wood / contaminated soil, development of targeted wood preservatives and genetic engineering of trees are the main objectives of research and development for biotechnology related to the wood preservation industry. Increasing the impregnability of refractory wood species by fungal pre-treatment has been successfully tested on pilot scale and full sapwood penetration has been achieved. To prevent bluestain or decay by using antagonistic microbial systems is the objective of biocontrol. Positive results have been achieved in the laboratory to control bluestain, however, these did not translate into success when applied in nature. Using ligninolytic enzyme systems and bacterial post-treatment has been shown to be a successful strategy to remediate creosote contaminated soil. The feasibility of this process largely depends on the technique of prior extraction of PAH from soil. Fundamental research into fungal decay mechanisms has revealed radical reactions based on transition metals to be possible key reactions for both brown and white-rot. This might offer a chance of developing targeted wood preservatives. Genetic engineering of trees has shown great progress in the past years. Developing wood with extended natural durability might be an option for the future, but as a prerequisite it will need more know-how on the molecular basis of natural durability.

1. Introduction

Biotechnology has been one of the fastest growing research areas in the past. Despite its considerable success in the medical sector biotechnological processes have been developed relatively slowly to convert lignocellulosic substrates. In the past decade, main progress was made in the pulp and paper industry where biotechnological processes have been successfully implemented and these have contributed to the constant improvement of technology of this industrial sector. Several new applications of enzymes have reached or are approaching the stage of commercial use. These include enzyme-aided bleaching with xylanases, direct delignification with oxidative enzymes, energy saving refining with cellulases, pitch removal with lipases, freeness enhancement with cellulases and hemicellulases as well as enzymatic slime control of

the paper machine (Viikari, 2002). Besides enzymes, biopulping the use of white-rot fungi to treat wood chips prior to refining in thermo-mechanical pulp production - is close to mill application. Energy savings of up to 30% during refining of wood chips and considerable strength improvements of resulting handsheets were achieved (Young and Akhtar, 1998). Fungal pretreatment of wood chips also resulted in reduced cooking time in sulfite pulp production (Messner, 1998). Recently these results have urged people to launch new research programmes to investigate the potential of biotechnology for improving fibre properties and binding capacity for producing fibre- and particle boards or wood composites. An overview on forest products biotechnology is presented in Bruce and Palfreyman (1998).

The objectives of biotechnological processes for the wood preservation industry differ largely from that of the pulp and paper and other wood industry. While in the pulp and paper and wood industry the modification of wood by, primarily depolymerizing enzymes is the main goal, wood should be prevented from becoming deteriorated by microbial enzyme systems in wood preservation. The complexity of this target might be the main reason why at present no biotechnological process has been implemented on industrial scale in the wood preservation industry. However, scientists have adopted this challenge and research is going on to solve the following tasks which are listed in the order of the status of their development with respect to the likeliness of being applied on commercial scale:

- Impregnability improvement of refractory wood species
- Biocontrol
- Bioremediation of treated wood / contaminated soil
- Development of targeted wood preservatives
- Genetic engineering of trees

2. Tools for wood biotechnology

Generally it has to be stated that biotechnological processes usually are only expected to become feasible if the aim of this process cannot be achieved by a chemical process. Usually, biotechnological processes are more expensive and additional training of personnel may be needed. Thus, the options for developing a chemical process have to be closely observed.

The main tools for wood biotechnology are either enzymes or fungi. Their main features making them interesting tools for wood biotechnology can be summarized as follows:

Enzymes:

- Biocatalysts, catalyzing only one specific reaction (with the exception of oxidative enzymes including “ligninases”)
- Glycoproteins
- Biodegradable
- Non-toxic
- Optimum temperature : 40-60°C

- Optimum pH : 4-8
- Reaction time: a few hours

Fungi:

- Optimum temperature: 20-30 ° (thermophilic fungi: 40-50°C)
- Optimum pH: 5-7
- Colonize interior part of wood
- Excrete enzymes (also in the interior part of the wood)
- Excrete biocontrol agents
- Disadvantages: non-specific reactions
  reaction time: > 1 week

Yeast and bacteria have been investigated to a lesser extent as they colonize primarily the wood surface. This may restrict their use rather for biocontrol applications.

When filamentous fungi are used for biotechnological processes, their hyphae colonize the wood surface relatively rapidly, depending on the density of the inoculum and supplementation with additional nutrients (Fig. 1). The wood rays function as the main entrance port and growth progresses fast via ray trachids, where parenchyma cells provide additional nutrient supply. From the ray cells hyphae spread into adjacent

Fig. 1: Colonization of the surface of a wood chip by hyphae of *Phanerochaete chrysosporium.*

vessels and find easy access to other vessels via pits (Fig. 2). Wood decay fungi additionally have the ability to penetrate wood cell walls by bore hyphae (Fig 3).

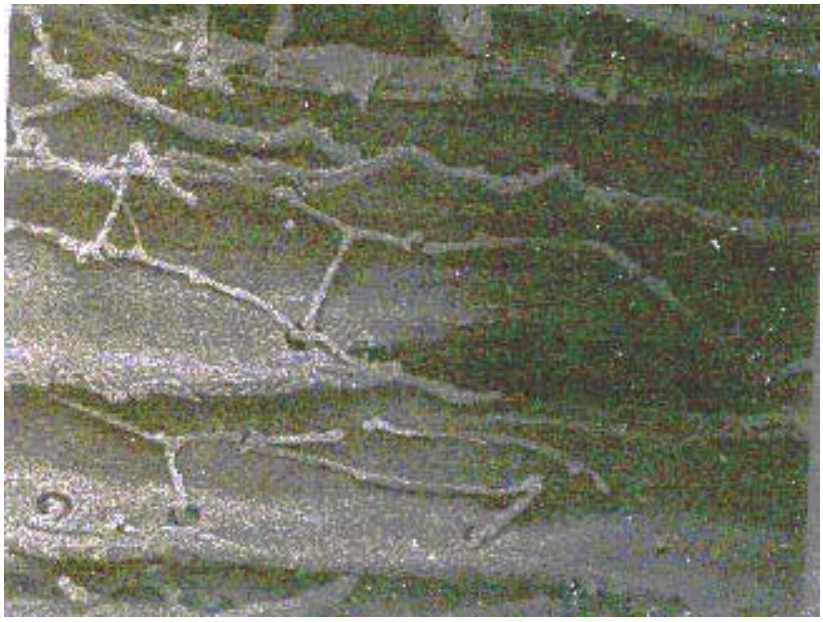

Fig. 2: Hyphae colonizing wood fibres via pits.

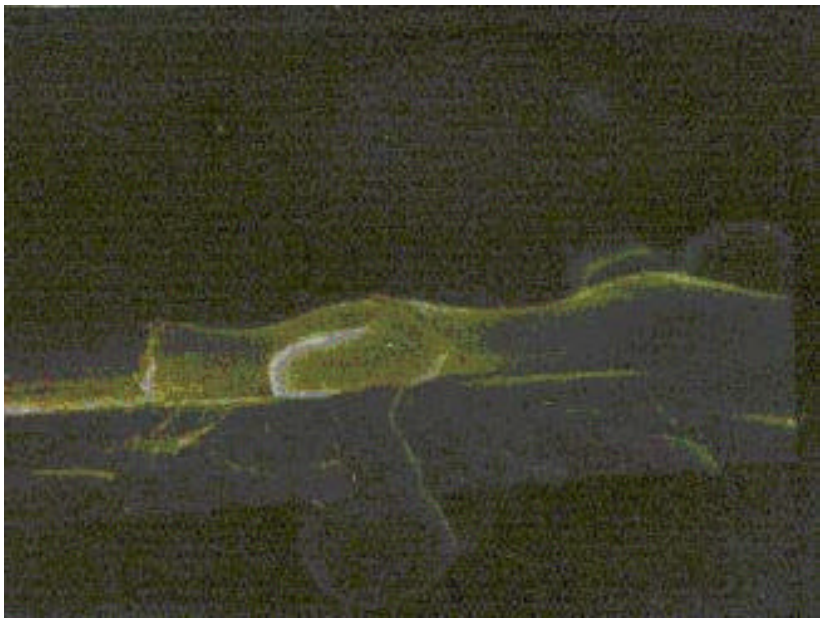

Fig. 3: Bore hypha penetrating a wood cell wall.

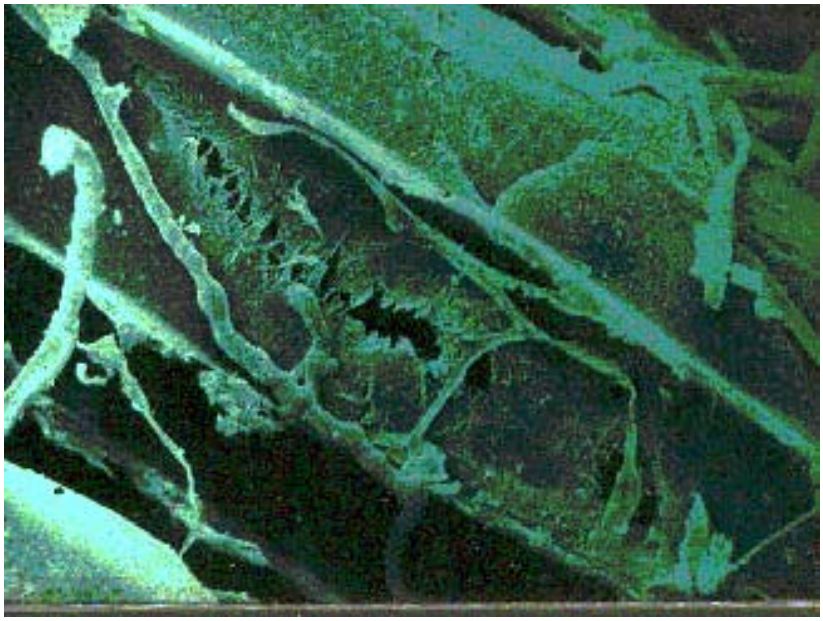

Fig. 4: Degradation of the wood cell wall by a white-rot fungus.

When colonizing the lumina of the wood fibres the hayphae start producing and excreting their enzyme systems and also other chemical compounds including antibiotics. As mentioned above, various enzymes partly induced by compounds of the substrate are excreted making the overall effect non-specific for a special substrate component (Fig 4).

For this reason it is of utmost importance to know exactly the reactions catalysed by a specific fungus with respect to the expected result of the biotechnological process. Among wood colonizing fungi, three main overall effects can be distinguished with respect to wood decay:

Mold fungi, soft-rot fungi:
Many of the mold fungi including *Trichoderma* species produce high activities of cellulases and hemicellulases but nevertheless are not able to attack the wood cell wall. Cellulose and hemicellulose in the wood cell wall is covered by lignin. Due to their high molecular weight, enzymes are not able to penetrate the pores of the lignocellulose complex to diffuse into deeper areas and degrade the structural elements (Srebotnik et al. 1988, Srebotnik and Messner 1990). For that reason fungi like *Trichoderma* can be used for permeability improvement or biocontrol while retaining full strength of the wood. However, a small group of molds - the soft rot fungi - are capable of attacking lignin and thus, lead to weight loss.

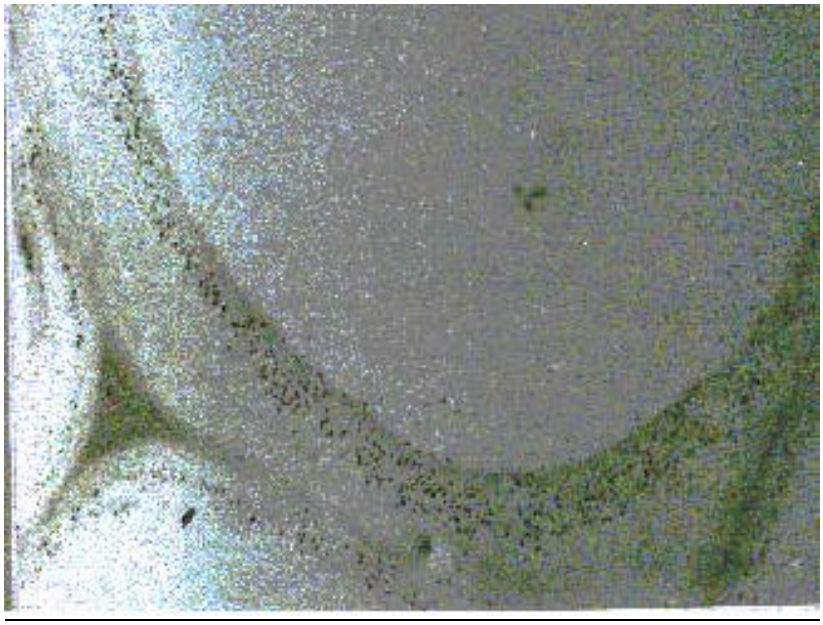

Fig. 5: Immunogold labelled ligninase enzymes of a white-rot fungus gradually penetrating the wood cell wall.

White-rot fungi:
These are the classical wood degrading fungi which are able to consume all components of wood. Their enzymes gradually penetrate the wood cell wall by degrading lignin and other components (Fig. 5) leading to weight- and strength loss. However, cell wall attack of some weakly wood degrading white-rot fungi starts only after 1-2 weeks colonization (Messner et al., 1998) which allows to use these fungi for biotechnological processes within a restricted time of cultivation.

Brown-rot fungi:
This group of fungi depolymerizes and consumes the carbohydrates rapidly, leading to immediate and heavy strength loss. In late stages of decay only the lignin part is left back.

3. Biotechnolgy for the wood preservation industry

Impregnability improvement of refractory wood species:

Many wood species including Douglas fir, Norway spruce, Sitca spruce and Fir are not fully commercially exploited because it is very difficult to achieve satisfactory penetration of preservative even when using pressure treatment systems. When the timber is seasoned, its permeability can be reduced to between 1-5% of that of green timber resulting in lateral preservative penetration of only a few mm. This is largely due to the aspiration of the pit membranes. Softwood of spruce becomes almost untreatable and heartwood is untreatable at all. However, according to EN 355-1, for commodities in Hazard Class 3, like external joinery such as windows, doors etc. minimum 6mm

lateral and 50 mm axial penetration of the preservative is required for instance in Nordic countries of Europe (NWPC Doc. No 1:1998). Full sapwood penetration is required for external cladding or garden timbers and especially for uses in Hazard Class 4, such as round wood in ground contact and additionally 6 mm lateral into exposed heartwood is required for sawn wood in ground contact.

The main technologies to overcome this problem are oscillating pressure methods and incising. After 15-20 hours oscillating pressure treatment of spruce or fir poles with CCB, copper penetration of 22 mm can be achieved (Graf and Bör, 1999). Incising is rather used for sawn wood (Morris et al. 1994). Petration of CCA has been reported up to 10-15 mm at an incision depth of 5 mm and an incision density of 7300# per m^2 (Anderson et al., 1995). Zahora and Hösli (1997) reported achievement of CSA and AWPA requirements for in ground use of Black spruce lumber after incising at a density of 9500# per m^2 and 4.5 h treatment. Although both methods can be applied to an extent where requirements for certain applications can be reached, methods leading to lower operating costs and increased qualities of treated wood would be of advantage for the wood preservation industry.

An old practice to use the potential of micro-organisms for impregnability improvement of wood is to store logs in ponds where the wood is colonized by bacteria, attacking the pit membranes. However, the results are very inconsistent. Positive effects on permeability reached by wood treatment with a mixture of cellulases and hemicellulases were reported by Militz (1993a, b). The improvement was not sufficient however to allow the development of a technical process.

A novel biotechnological method based on the pre-treatment of wood with fungi selected from a wide array of strains has been developed in a joint effort by the two companies LIGNOCELL Wood-Biotechnology GmbH of Austria and TRIPERM Processes Ltd. of Scotland. It improves permeability of refractory wood species considerably (Rosner et al., 1998; Tucker et al. 1998). Two types of fungi, i) either wood colonizing molds like specific strains of *Trichoderma* or ii) weakly wood degrading basidiomycetes were chosen for permeability enhancement. The pre-treatment process includes inoculation of wood having a moisture content above 50% with fungal inoculum containing a nutrient solution and subsequent incubation. When using weakly wood degrading basidiomycetes, prior decontamination of the wood surface by short steaming is recommended, furthermore, eradication of the fungi by subsequent heat treatment is needed. After incubation of 4 weeks with *Trichoderma* or of 1 week with type ii) fungi and subsequent pressure treatment with creosote GX+ in a conventional Rueping process, full sapwood penetration of spruce logs was achieved (Figs.: 6; 7; 8)

Fungi	Incubation time weeks	Retention kg/m^3	Strength loss %
T. viride	4	80	0
T. aureoviride	4	70	0
Dichomitus squal.	2	90	10-15
	3	150	10-15
Phanerochaete chr.	1	110	0
	2	140	10

Table 1: Effect of fungal pre-treatment of spruce logs on retention of creosote GX+ and strength loss.

In a new series of experiments with spruce log sections carried out by Ed Tucker and Alan Bruce, University of Abertay, Dundee / TRIPERM Processes Ltd. (unpublished results), with *Trichoderma aureoviride* and 4 weeks incubation time demonstrated that:

- Decontamination of logs is not needed when *T. aureoviride* is used.
- Full penetration of spruce sapwood was achieved at incubation temperatures of 7°C, 16°C and 22°C.
- Moisture content of higher than 50% is needed to allow fungal penetrability improvement.

The advantage of using basidiomycetes like the selected strain of *Phanerochaete chrysosporium* is the short incubation time needed and the option to extend penetrability also to the heartwood area. However, prior decontamination of logs and eradication after the fungal pre-treatment has to be considered. If the incubation time is kept at 1-2 weeks, no or very little strength loss was measured (Table 1). For reasons explained earlier, no strength loss has to be expected when strains of *Trichoderma* are used.

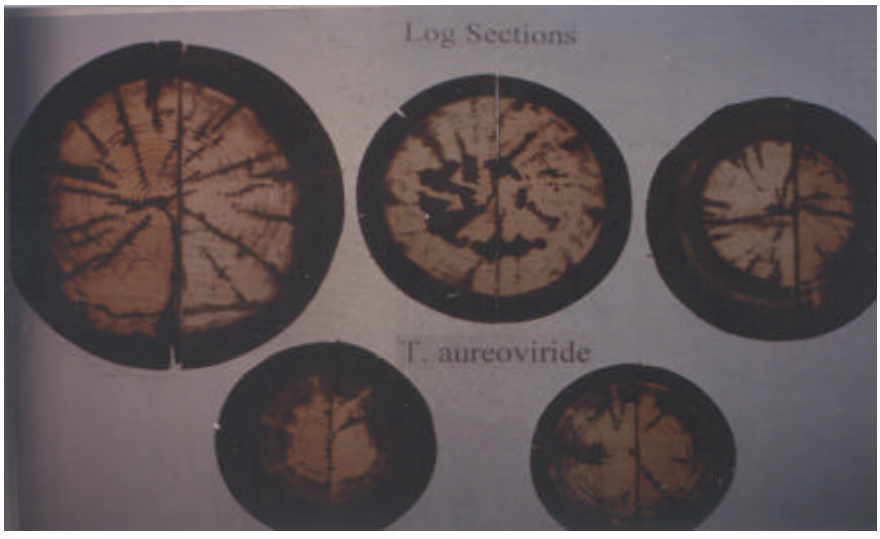

Fig. 6: Penetration of creosote after pre-treatment with *Trichoderma aureoviride*

Fig. 7: Penetration of creosote after pre-treatment with *Dichomitus squalens*

Fig. 8: Penetration of creosote after pre-treatment with *Dichomitus squalens.* The selected basidiomycetes also effected heartwood penetration.

These results demonstrate that fungal pre-treatment of refractory wood species considerably improves its treatability and has a high potential to be used in the wood preservation industry. Patents for the process are pending in EU, USA and Canada.

Biocontrol

Fungi and bacteria acquired very complex and efficient strategies to compete other micro-organisms in their struggle for substrates. Using antagonistic living micro-organisms in order to prevent deterioration of goods by other micro-organisms is a method known as biocontrol. Especially when legislation becomes increasingly restrictive with respect to eco-toxicology, using antagonistic micro-organisms and investigating their chemical interaction to develop new environmentally more benign biocides is an interesting strategy. On a restricted scale biocontrol is established already in agricultural systems. It has been successfully used against *Rhizoctonia* damping off, grey mold on fruits, post-harvest rot on apples or soil borne plant diseases. Although many fungi including yeasts and filamentous fungi and also bacteria were identified to exert biocontrol activities, most work has been done using strains of the ubiquitous fungus *Trichoderma.* Its complex mechanism is understood relatively well. Synergistically interacting components (Fig. 9) of this mechanism are:

- Competition for nutrients: fast growth, efficient extracellular enzyme systems, siderophores to scavenge metals
- Volatile antibiotic compounds: 6-pentyl-á-pyrone
- Soluble antibiotic metabolites: trichothecenes, pentaiboles
- Mycoparasitism by lytic enzymes: chitinases, glucosidases

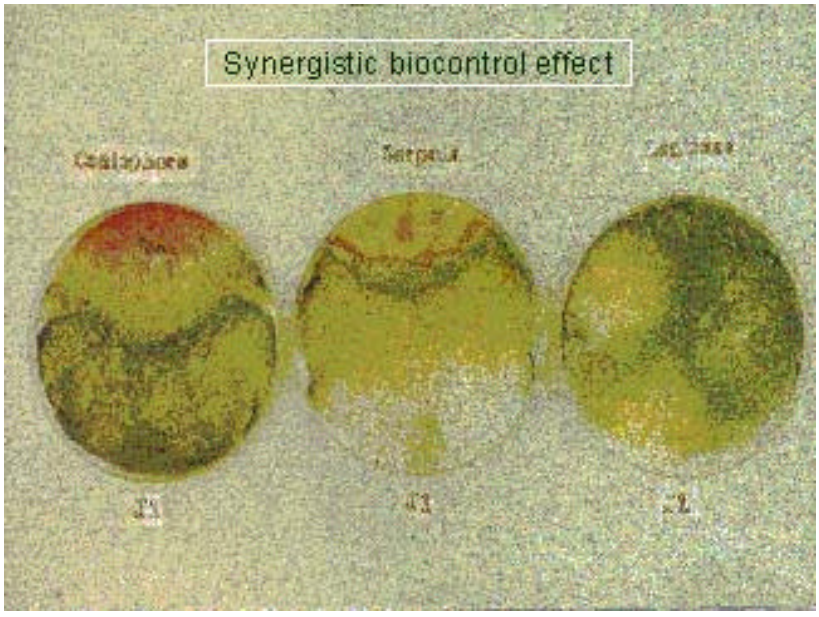

Fig. 9: Synergistic biocontol effect of a selected strain of *Trichoderma harzianum* on three wood decay fungi on malt agar. The target fungi are overgrown by *T. harzianum.*

Although biocontrol has been established in agricultural systems, and many attempts have been made to use this technology for wood preservation, results are restricted to laboratory work or pilot scale experiments. (A comprehensive overview on research is given in Freytag et al. (1991), Bruce (1998)). This is due to a number of additional challenges the prevention of wood bioteterioration provides including: length of protection; diversity of target (brown-rot, white-rot, soft-rot, blue-stain); efficacy of control system.

Biocontrol of blue-stain:
Compared to control of decay, the time span during which control has to be maintained is relatively short which might favour the first successful biocontrol product to be developed in this area. The main strategies for product development are focussing on:

- Screening of efficient biocontrol strains including, filamentous fungi, yeasts and bacteria.
- Developing albino strains of *Ophiostoma piliferum* (Farrell, 2001).
- Integration of biocontrol organisms into biocides
- Inhibition of melanin production

Significant stain reduction was achieved in the laboratory (Morrell and Dickinson, 1998). However these results did not translate into field success and more research is needed to better understand the factors responsible for a successful biocontrol product. Some of them are:

- Effect of ecology
- Range of sensitivity of target fungi
- Components of an effective biocontrol sytem and its synergies
- When are these components produced during growth of the micro-organisms on wood?
- How to boost biocontrol efficacy?
- What are the defence mechanisms employed by the target fungi?

Biocontrol of decay:
To prevent decay by biological means is very tough challenge. First of all, the target organisms include brow-rot, white-rot and soft-rot fungi, belonging to basidiomycetes as well as ascomycetes employing different decay mechanisms and also responding differently to biocontrol agents. The second serious problem to be tackled is the length of protection required. In case of palisades or poles it might be up to ten or even 30 years. A more attractive area of application for biocontrol systems seems to be to protect freshly felled logs from infection by decay fungi until preservative treatment.

Although the challenge is tough, some results are encouraging to continue studying biocontrol of decay. Bruce and King (1991) reported that 7 years after pole inoculation with a strain of *Trichoderma*, wood was resistant to attack by decay when wood blocks of this poles were submitted to decay fungi in the laboratory. This long term protection effect might be explained by the fact that many fungi produce resting spores called chlamydospores within their mycelium. These are able to survive for extremely long periods of time in dormancy if moisture content of the substrate is too low.

Consequently, a strategy for long term protection would be to inoculate wood with a high density fungal inoculum, leading to a high number of chlamydospores produced by *Trichoderma* inside the wood. If moisture content of the wood rises above its minimum concentration, the spores would germinate and create new mycelium to protect wood against prospectively invading decay fungi.

To investigate this hypothesis a pilot experiment was carried out by the author (unpublished results). Strains of *Trichoderma* were isolated and screened according to EN 113 for their performance against wood decay fungi. *Trichoderma harzianum* LC1 (Fig.: 10) was selected and investigated for its toxicity by an authorized test institute (Laboratory of Pharmacology and Toxicology, Hamburg). As all toxicity tests were negative (Table 2), the strain was chosen for a pilot experiment.

50 commercial pine poles (P. radiata) were inoculated with a spore suspension (100 000 / ml) by dipping, no nutrients were supplemented, the poles were incubated for 4 weeks at ambient temp. Fungal growth was controlled by light- and electron microscopy. Fungal mycelium and chlamydospores as well as konidiospores were found to be produced in the sapwood (Fig. 11).

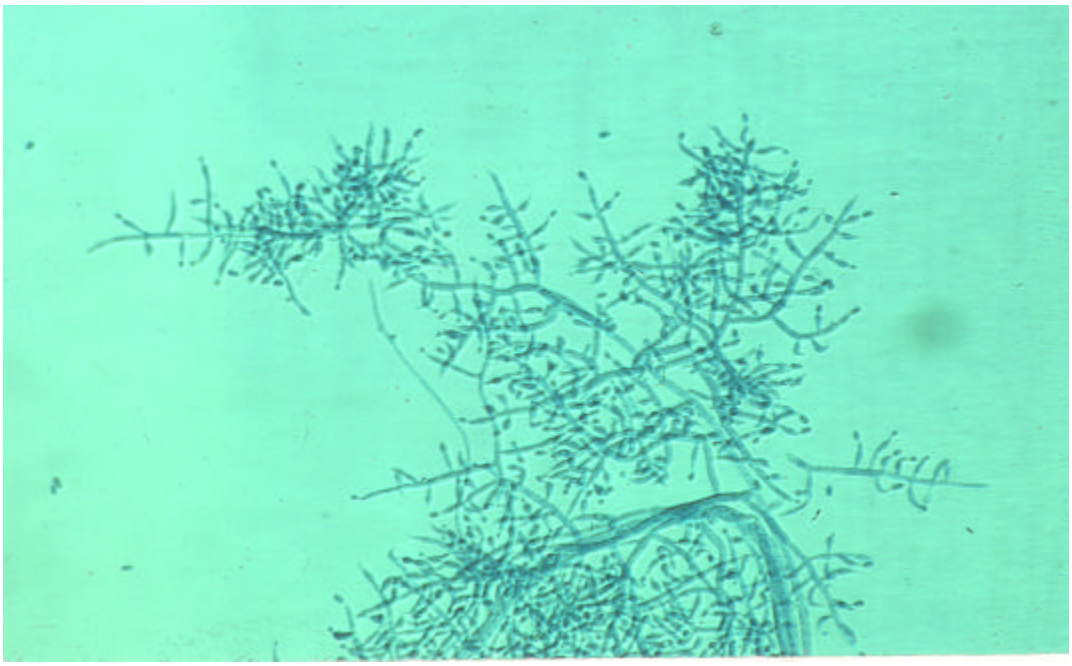

Fig.: 10: *Trichoderma harzianum* LC1

Table 2: Toxicity of *Trichoderma harzianum* LC1 spore suspension

Acute oral toxicity	no intolerance reactions
Acute dermal tox.	no intolerance reactions
Acute skin tolerance	no irritating properties
Acute eye irritation	no irritating properties
Skin sensitation	no sensitising properties
Ames-test	no mutagenic effect

The performance of the logs was investigated under conditions of hazard class 4 when planted into soil and under conditions of hazard class 3 when screwed on concrete mast feet (Fig.: 12).

Result: After 3 years of exposure the first poles planted in soil started to fail which was comparable to the controles. The poles screwed on concrete mast feet performed well and showed no signs of fungal attack after 8 years of exposure. Investigations of the fungi colonizing the badly performing poles in ground contact demonstrated that the main type of attack was soft-rot attack which was only weakly inhibited by the selected strain of *Trichoderma*. The result shows that development of strains protecting against a broader spectrum of target fungi is needed for this purpose.

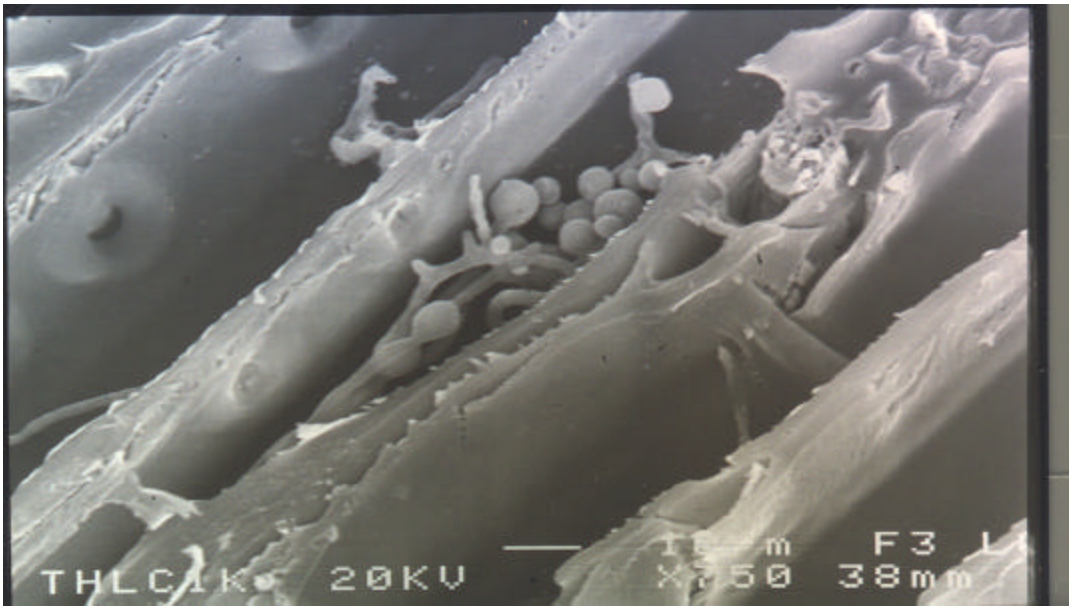

Fig. 11: Chlamydospores of *Trichoderma harzianum* produced in the sap-wood of pine poles after inoculation.

Fig. 12: Spruce poles treated with *Trichoderma harzianum* planted in soil and screwed on mast feet, respectively.

Bioremediation of creosote treated wood and contaminated soil

Polycyclic aromatic hydrocarbons (PAH) are the active components of creosote. Especially some of the components composed of 4 to 6 rings such as benzo[a]pyrene were found to be cancerogenic. PAH are hydrophobic and tightly bound to the soil matrix which decreases their bioavailability considerably. Another reason for their recalcitrant nature is that compounds containing 4-6 rings are hardly be degraded by soil bacteria. Thus, PAH can be detected in contaminated soil decades after they were introduced by former treatment plants of low environmental standards, posing an environmental hazard. Creosote treated railway ties and transmission poles at the end of their service life are a further source of creosote containing waste. However, as long as incineration plants meet the required standards, creosote treated waste-wood becomes a valuable energy source and there is no need to develop bioremediation strategies for its decontamination..

PAH contaminated soil poses a much larger problem due to the high costs of incineration. and produces a biologically inactive end product. Extraction of PAH by solvents or soil washing techniques and subsequent incineration or land farming ofsludges is state of the art, but is not widely used, due to high costs. The method of choice would be to degrade and detoxify PAH by a bioremediation technique. Principally, this can be reached by using white-rot fungi (Messner, Böhmer, 1998). However, as the natural substrate of these organisms is wood, they do not compete well with other organisms in soil. This problem can be avoided when using isolated ligninolytic enzymes. A three-step process comprising i) PAH extraction by soil washing ii) oxidation of extracted PAH by an oxidative enzyme system and iii) post-

treatment by adapted bacteria proved to be very efficient in reducing the concentration of PAH in soil (Böhmer et al. 1998). The oxidative enzyme system is based on laccase and two mediator compounds such as HBT and an unsaturated fatty acid (Fig. 13). High molecular weight PAH are oxidized by the laccase system to low molecular weight compounds which in turn can be converted to CO_2 by an adapted bacterial population. As shown in Fig. 14, all 4-6 ring PAH (underlined) of the 14 EPA PAH, which resist bacterial decay, were depolymerised by the enzyme system alone. After a bacterial post-treatment step all of the 14 PAH were degraded, demonstrating the high potential of this system to be used for bioremediating PAH contaminated soil. When used on soil, the critical step is to find an efficient and feasible extraction technique.

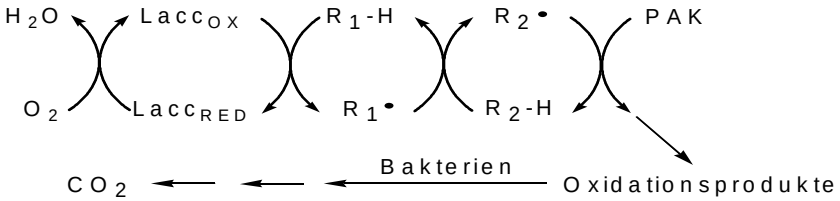

Fig. 13: Oxidative enzyme system comprising laccase and two mediating compounds (R_1, R_2) to degrade PAH and subsequent conversion of oxidation products by bacteria to CO_2.

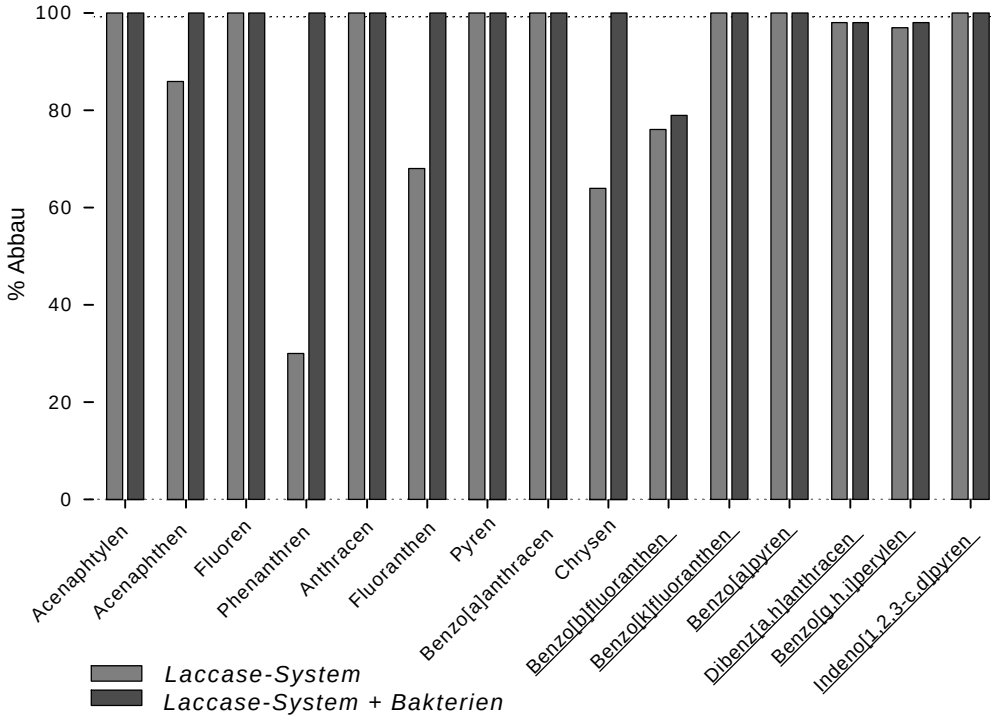

Fig. 14: Degradation of 14-EPA PAH by the laccases system alone and the laccases system plus bacterial post-treatment, respectively.

The targeted wood preservative approach

White-rot and brown-rot fungi are the most active wood degrading organisms and thus, they play a key role in the carbon cycle on earth. Some species are known to cause heavy damage to construction and building materials requiring that this damage be prevented by wood preservatives. Chemical products to increase wood durability currently in use are very effective but a strong demand to develop new products with less environmental impact cannot be overlooked. Understanding the microbial mechanisms leading to wood degradation is a prerequisite for understanding both the development of new wood preservatives as well as wood biotechnology processes. It is generally accepted that low molecular weight compounds, smaller than enzymes, are involved in brown- and white-rot. For brown-rot a Fenton-type system empoying iron as catalytic transition metal has been proposed (Xu and Goodell, 2001).

For white-rot a new, powerful lignin degrading system based on coordinated copper and peroxide, either hydrogen peroxide or organic peroxides, has been proposed to be the agent involved at least in the initial depolymerization and degradation of lignin (Watanabe et al. 1998; Fackler et al. 2002; Messner et al 2002). The capacity of the copper system to degrade native wood lignin was evaluated by two methods: i) a newly developed method employing section staining and ii) UV-microscopy. It was shown that the wood cell walls of hardwood were almost completely delignified and the middle lamellae was degraded after treatment with coordinated copper and organic hydroperoxides (Fig. 15) (Lamaipis et al, 2000). The effect matches the effect of selective white-rot fungi.

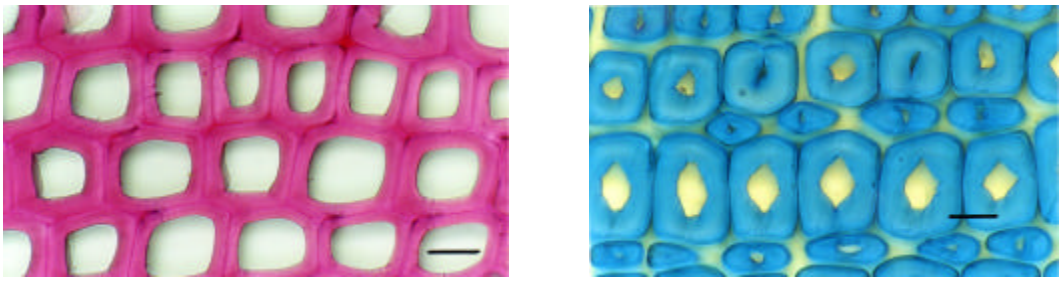

Fig. 15: Sections of pine sap-wood, stained with safranin / astra blue. Left: control, lignin is stained red. Right: delignified by coordinated copper plus organic peroxide. Section is delignified, cellulose is stained blue. (bar: 20 μ).

With H_2O_2 only, the cell walls of hardwood were degraded to some extent, while the middle lamella was not attacked. It is assumed that the coordinated copper system is involved in white-rot decay and is probably functioning as the agent primarily responsible for selective white-rot.

The copper system seems to be related to the brown-rot mechanism, both of them are employing radical reactions catalyzed by transition metals. Inhibiting this mechanism may offer a chance to develop new targeted wood preservatives.

Extended natural durability

Genetic engineering of trees has made great progress during the last years. The main focus is related to pulp and paper production where trees containing less lignin, and furthermore containing lignin of a chemical structure, which can be dissolved more easily during chemical pulping, will be of great advantage. Remarkable results are reported in the literature (Chiang, 2001). Birch trees were developed, containing approx. 50% less lignin at an increased amount of cellulose. Furthermore the lignin composition was changed in a way that the cooking time could be decreased.

Considering these results, it can be assumed that genetic engineering will also offer the chance to change the chemical composition of trees in a way that their natural durability will be improved. However, detailed know how on the chemical and molecular basis of natural durability is a prerequisite and fundamental research in this field is needed.

4. Conclusion

Biotechnological processes for converting lignocellulosic substrates have been implemented successfully in pulp and paper production. Biotechnology related to wood preservation is not that far developed, however, good progress has been made and impregnability improvement of refractory wood species by fungal pre-treatment is close to large scale application. Positive results have been achieved in the laboratory to control bluestain, however, these did not translate into success when applied in nature and more research into the basic principles of microbial antagonisms is needed to make this most interesting approach applicable for the wood preservation industry. Using ligninolytic enzyme systems and bacterial post-treatment has been shown to be a successful strategy to remediate creosote contaminated soil. The feasibility of this process largely depends on the technique of prior extraction of PAH from soil. Fundamental research into fungal decay mechanisms has revealed radical reactions based on transition metals to be possible key reactions for both brown and white-rot. This might offer a chance of developing targeted wood preservatives. Genetic engineering of trees has shown great progress in the past years. Developing wood with extended natural durability might be an option for the future, but as a prerequisite it will need more know-how on the molecular basis of natural durability.

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