

Bioremediation of Waste Copper/Chromium Treated Wood Using Wood Decay Fungi

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The expected service life of copper/chromium (CCA or CCB) treated wood is about 25–50 years. After this period, the treated wood is discarded. However, due to the toxic nature of the elements in such treated wood, uncontrolled incineration and landfill or disposal into the environment are not considered environmentally friendly management solutions. A combination of bioremediation, extraction, and recycling of the preservatives from the waste wood is a much more promising and environmentally acceptable management solution, which is based on the conversion of fixed biocides in the wood into soluble forms, which can subsequently be leached out of the wood.

In order to elucidate the mechanism of this process, copper/chromium treated Norway spruce (*Picea abies*) wood samples were leached after exposure to copper tolerant (*Antrodia vaillantii* and *Leucogyrophana pinastri*) and copper sensitive (*Gloeophyllum trabeum* and *Poria monticola*) wood decay fungi. Furthermore, the ability of the mycelia of these fungi to penetrate and overgrow the wood blocks was investigated by using small sticks of non-impregnated-control wood ($r = 1.5$ mm, $l = 25$ mm) inserted into a hole, bored into the center of the treated wood samples, and sealed with epoxy sealer. Sterilized, leached, and non-leached impregnated and unimpregnated wood specimens were exposed to brown rot fungal strains for 1, 2, 4, or 8 weeks. After the respective exposure periods, the inserted wood pieces were removed from the specimens and put onto nutrient medium in petri dishes. Growth of

the fungal mycelia from these pieces was visually examined. Rate of colonization was also determined by measurement of CO₂ production. The fungal growths were stimulated by immersing the specimens into aqueous solution of glucose or corn steep liquor prior to exposure to the fungi. After exposure, the specimens were leached and the concentrations of copper and chromium leached were determined. EPR measurements of the leached and non-leached samples were performed in order to determine the paramagnetic complexes that were formed. This method enabled us to characterize structural changes of copper and chromium in the wood.

The fastest colonization of impregnated wood was by the copper-tolerant *Antrodia vaillantii*. The addition of nutrients onto the surface of wood specimens increased colonization by the fungi. All wood decay fungi investigated (copper-tolerant as well as copper-sensitive) increased heavy metals leaching from the treated wood. These fungi were found to have influenced the de-fixation of the biocides via oxalates formation. The EPR measurements indicate that the transformation of copper into copper-oxalate by the fungi was essential but not the only mechanism responsible for copper-tolerance by these fungi. However, our results also show that other acids, such as acetic and malic acid, were also responsible for the increased copper and/or chromium leaching. These results are important in elucidating copper toxicity by wood decay fungi and for using these fungi for bioremediation of treated wood wastes.

Degradation of Dioxin and PCB by White-Rot Fungus *Phlebia brevispora*

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Polychlorinated biphenyls (PCBs) consist of 209 different congeners and represent a family of compounds with a wide range of industrial applications in heat transfer fluids, di-electric fluids, hydraulic fluids, flame retardants, plasticizers, solvent extender, and organic diluents. The high resistance of these toxic compounds requires drastic conditions for decomposition, either high temperatures or chemical reagents, both very expensive processes. Some PCB congeners have been shown to be transformed by both aerobic and anaerobic bacteria. The aerobic biodegradation of PCBs is generally limited to less-chlorinated congeners (5 or fewer chlorines per biphenyl molecule) by a mechanism involving dioxygenation of the aromatic ring. The more-chlorinated congeners are generally recalcitrant to aerobic degradation. To degrade PCBs, we focused on the white-rot fungi. The degradation of PCBs by white rot fungi, wood-degrading Basidiomycetes has been known since 1985 (Bumpus et al., Science, 1985, 228: 1434-1436). Although many fungi have been tested for their ability to degrade PCBs, including the white rot fungi, the mechanism of PCB biodegradation has not been definitively determined for any fungi.

White-rot fungus *Phlebia brevispora* TMIC33929 can degrade some of the polychlorinated dibenzo-*p*-dioxins (PCDDs); therefore, this fungus is useful in examining the bioremediation potential of white-rot fungi. When coplanar PCB mixture (Co-PCBs) containing 12 PCB congeners (#77, #81, #105, #114, #118, #123, #126, #156, #157, #167, #169, #189) were used in the degradation experiment as the substrate, 3,3',4,4'-tetraCB (#77), 2,3,3',4,4'-pentaCB (#105), 2,3',4,4',5-pentaCB (#118), 3,3',4,4',5-pentaCB (#126), and 2,3',4,4',5,5'-hexaCB (#167) were degraded by *Ph. brevispora*. By GC/MS analysis, *meta*-methoxylated metabolites were detected from

the culture with these compounds. To investigate the metabolic pathway, 4,4'-dichlorobiphenyl (4,4'-DCB) was treated with some white-rot fungi. The metabolic pathway of 4,4'-dichlorobiphenyl (4,4'-DCB) was elucidated by the identification of metabolites upon addition of 4,4'-DCB and its metabolic intermediates. Hydroxylation and methoxylation of substrate were observed in the culture with *Ph. brevispora*, *Phanerochaete chrysosporium*, and *Phanerochaete* sp. MZ142. However, hydroxylation positions of 4,4'-DCB by each fungus were different. In the case of *Ph. brevispora*, 4,4'-DCB was initially hydroxylated to 3-OH-4,4'-DCB. The metabolic pathway of 3-OH-4,4'-DCB was branched to form 3-OCH₃-4,4'-DCB and to form 4-chlorobenzoic acid, 4-chlorobenzyl alcohol, and 4-chlorobenzaldehyde. Degradation pathway of 4,4'-DCB by *Ph. brevispora* showed a similar reaction to that by *Phanerochaete* sp. MZ142. The pathway to form 3-OCH₃-4,4'-DCB occurred first and *s*-adenosylmethionine (SAM)-dependent methyltransferase was involved in this biotransformation. 3-OCH₃-4,4'-DCB was degraded further through hydroxylation. These results indicate that hydroxylation and further methylation are the main pathways involved in degradation of PCBs by white-rot fungi.

To examine the bioremediation potential of *Ph. brevispora* in dioxin contaminated soil, *Ph. brevispora* was inoculated to the sterilized soil that was contaminated with 2,7-dichlorodibenzo-*p*-dioxin or 1,3,6,8-tetrachlorodibenzo-*p*-dioxin. *Ph. brevispora* can grow in the soil environment and can degrade each compound. Although the growth of the fungus was improved with organic-rich soil, this degradation was strongly inhibited in organic-rich soil. These results indicate that the existence of organic material was the main inhibition factor of bioremediation potential of white-rot fungi in soil condition.

Biodegradation Study of Polycyclic Aromatic Hydrocarbons (PAHs) and Polychlorinated Phenols (PCPs) by *Hypoxylon fragiforme* and *Coniophora puteana*

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White rot fungi and brown rot fungi are known to degrade structural components of wood; lignin is decomposed primarily with extracellular enzymes of white rot fungi, while hemicellulose and cellulose are degraded by brown rot fungi.

In the present work, a comparative biodegradation study of two sets of compounds: polycyclic aromatic hydrocarbons (PAHs) and polychlorinated phenols (PCPs), was conducted with two wood degrading fungi: *Hypoxylon fragiforme* (white rot) and *Coniophora puteana* (brown rot). The selected sets of pollutants are structurally similar to the lignin component of the wood and can be degraded or bio-transformed primarily with white rot fungi and to lesser extent with brown rot fungi, enzymatic systems.

Biodegradation of 12 PAHs (acenaphthene; acenaphthalene; anthracene; 1,2-benzoanthracene; benzo(k)fluoranthene; biphenyl; fluorine; 1-methylfluorene; 1-methylnaphthalene; 2-methylnaphthalene; fenantrene; pyrene) and eight PCPs (3,4-dichlorophenol; 3,5-dichlorophenol; 2,3,6-trichlorophenol; 2,4,5-trichlorophenol; 2,4,6-trichlorophenol; 2,3,4,6-tetrachlorophenol; 2,3,5,6-tetrachlorophenol; 2,3,4,5,6-pentachlorophenol) was studied, each in nine concentrations (0.078, 0.156, 0.313, 0.625, 1.25, 2.5, 5, 10, and 20 mmolL⁻¹). The biodegradation/biotransformation of selected pollutants was performed separately by *H. fragiforme* and by *C. puteana* in liquid media (2% aqueous solution of malt extract and 0.5% solution of glucose). After 20 days of incubation, the products of transformation were extracted from the aqueous media with non-polar solvent benzene, and the level of nondegraded pollutants was determined using the gas chromatography/mass spectrometry (GC/MS) method. Intermediates of the degradation process were identified by applying a database within the MS system. In order to find reasons for differences in the levels of

degradation of pollutants with two selected ligninolytic fungi, the QSAR approach was used. QSAR analysis was performed only with PAHs because the number of polychlorinated phenols was too small for reliable QSAR analysis. QSAR analysis enables searching for correlations between the level of biodegradation of pollutants and their structural descriptors. The best QSAR model for biodegradation of PAHs with white rot fungi (*H. fragiforme*) was obtained with three descriptors: two topological (IDE and HVcpx) and one physical-chemical descriptor, the molar mass of the compound. The results were explained by substitution of molar mass as a descriptor with the quantum-mechanical descriptor - relative energies of π -electrons of selected PAHs molecules. Statistical parameters of the new model were within the range of those obtained with the best model in which molar mass was used as a descriptor. Based on these results we concluded that the reactivity of PAHs towards the attack of radicals in the first step of degradation depends on the relative energy of π -electrons of the molecules (the higher the relative energy, the lower the reactivity of the molecule towards hydroxyl and other radicals), and their geometry which is reflected in the topological descriptors. QSAR study of biodegradation levels with the brown rot fungus *C. puteana* gave the best QSAR model if solubility in water, number of hydrogen atoms in the molecule, and energy of the lowest unoccupied molecular orbital (LUMO) were used as molecular descriptors. In this case, topological descriptors did not correlate with the biodegradation level; therefore, it can be inferred that enzymes of the brown-rot fungus *C. puteana* are less specific than those of white rot fungus *H. fragiforme*. Chlorinated phenols (as in the concentration range of this study) were totally transformed into intermediates after 20 days of incubation with the white rot fungus *H. fragiforme*, while with

the brown rot fungus *C. puteana* biotransformation of tetrachlorophenol and pentachlorophenol was below 60%. Biotransformation levels of chlorinated phenols

with the brown rot fungus *C. puteana* depended on the number of chlorine atoms and their relative position to the hydroxyl group in the molecule.

The Mycoremediation of the Degraded Surfaces by *Pleurotus ostreatus*

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The exploitation of fungal capacity to improve unproductive soil was named mycoremediation. In our investigation, mycoremediation is used for reconstruction of degraded surfaces; for example Šoštanj Thermal Power Plant (TEŠ) ash dump and dolomite cut slope of the forest road. The goal is to enrich organically poor and damaged surfaces by inoculating beech chips with white rot fungi and, consequently, prepare these surfaces for reforestation. In this paper we will present only the experiment on the ash dump. Furthermore, we will concentrate on the laboratory experiment testing wood degrading ability in the presence of contaminated substrates.

Six strains of *Pleurotus ostreatus* were used in quick screening for the beech degrading ability with the mini-block method. Fungal strains AG PLAB, AG PloS2, and AG Plo3 were obtained from the collection at the Institute of Natural Sciences, Ljubljana, and ZIM 76, ZIM 1002, and ZIM 1006 from the ZIM collection at the Biotechnical Faculty, Ljubljana.

The modified mini-block method was used to test the wood degrading ability of *P. ostreatus* ZIM 76 in the presence of contaminated substrates, composed from the beech wood chips and corresponding concentrations of the fly ash (0%, 5%, 10%, and 20% according to the chip mass), which was obtained at TEŠ. The mini-block samples were exposed to mycelium on the contaminated substrate for 10 weeks. The pH values of the contaminated substrates and the mass loss of mini-blocks were measured after the experiment.

The fungal spawn consisted of wood material, wheat grains, and mycelium of *P. ostreatus* ZIM 76. The 900 m² large abiotic site poor with organic matter and severely contaminated with heavy metals at TEŠ ash dump was prepared. The experimental

field was enriched with wood chips with added hydrogel. In 2006, 2% inoculation with spawn made on wood substrate on selected experimental areas was executed.

Three months after the inoculation the fruiting bodies from one square meter (3 repetitions) were collected, dried (60°C, 72 h), and weighed. The yield was calculated on fresh wood chip mass. The detection of the heavy metals in the collected fruiting bodies is in the mode of proceeding.

The screening test revealed that the fungal strain ZIM 76, among the strains primarily isolated from the deciduous trees caused the highest beech decay rate (19.3% ± 6.6%) after 8 weeks of exposure time.

The incubation of wood samples on the contaminated substrates overgrown with *P. ostreatus* ZIM 76 mycelium showed the highest wood mass loss (23.9% ± 8.3%) in the control experiment and the lowest wood mass loss (9.0% ± 2.4%) at 20% fly ash concentrations. The pH values increased with the fly ash concentrations from 4.5 to 6.0.

The fungal spawn was successfully prepared and inoculated at the test fields and 3 months after the inoculation abundant fruiting of oyster mushrooms appeared. The yield after 3 months was (2.1 ± 0.1) kg of the fruiting bodies dry mass per m³ of fresh wood chips.

Pleurotus ostreatus was chosen for the inoculation because it is easy to grow, highly adaptable, and competitive with other fungi. Heavy metals are toxic when present in excess; they can inhibit growth, cause morphological and physiological changes, and also affect fungal reproduction. They also influence extracellular enzymes at the level of transcription and during enzyme action. The visual observation revealed

that mycelium growth depended on the concentration of the added fly ash. Furthermore, the mass losses of wood samples are shown to be in correlation with the fly ash concentration. As stated above, on the one hand, we can conclude that different heavy metals present in the fly ash interfere with fungal ability to grow and degrade wood components. But on the other hand, we cannot neglect the influence of pH, which was slightly higher with the addition of fly ash material. In the *in vivo* part of our experiments (test field at TEŠ ash dump) we expect that the spreading of the inoculated *P. ostreatus* mycelium will compete with the naturally appearing fungal species. The wood decay and consequently the formation of the organic soil components and natural succession are expected to be accelerated. With planting trees on research areas, the additional factor influencing soil alteration is added. The rhizosphere and the surrounding soils

are reported to vary in chemical, physical, and biological characteristics. The rhizosphere is rich with organic substances, which are mainly root exudates, and with different organisms among which numerous interactions take place. This enables the rhizosphere to enrich the soils with organic compounds, microbial and fungal communities, such as mycorrhiza, and also enables it to reduce the influence of the pollutants.

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Fungal Biodegradation of Plastics and Soil Pollutants

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Fungi is unique group of organisms capable of secreting various enzymes that enable them to colonize a wide range of living or dead tissues including plants, wood, leaf litter, soil, and compost. Ligninolytic Basidiomycetes (a group of fungi into which most of the cultivated and medicinal mushrooms belong) have developed a specific mechanism for the degradation of recalcitrant compounds such as lignin. The research on biodegradation of synthetic polymers and different recalcitrant pollutants contaminating soil is the topic of the lecture, as well as the use of the composting method for biodegradation tests.

The research on biodegradation of soil pollutants by ligninolytic fungi had its beginning in the USA in 1985 when the ligninolytic fungus (LF) *Phanerochaete chrysosporium* Burds. was found to be able to degrade, besides the lignin macromolecule, many toxic substances contaminating the environment such as chlorophenols, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls and dioxins, syn-

thetic dyes, different pesticides, and others. Since 1990 we have screened a large collection of LF for ligninolytic activity and biodegrading potential. We developed a step-by-step process to select the most promising candidate to be used in soil bioremediation (mycoremediation). Individual requirements (decolorizing potential, growth parameters, ability to degrade respective pollutants, ability to colonize non-sterile toxic soil) that the fungus should possess are evaluated from the first screening to application in practice are discussed in the lecture.

Field application of fungi in soil remediation is not always successful. More research is still needed to establish mycoremediation as an affective and reliable soil-remediation technology. Both positive and negative field-scale experiences of fungal treatment of polluted soil using mycelia of *Pleurotus ostreatus* (Jacq.: Fr.) P. Kumm., *Phanerochaete chrysosporium*, and other *Phanerochaete* species that have been performed in the Czech Republic, Germany and the

USA are discussed in the lecture. Special concern is given to the use of spent mushroom compost for mycoremediation.

Composting of contaminated soil has been used to treat solid waste such as agricultural wastes, sewage sludge, and food wastes. The technique we also used for bioremediation of PAH-contaminated soils originating from different industrial sites. Composting matrices are rich sources of microorganisms including bacteria, actinomycetes, and fungi that can degrade the pollutants. To standardize experimental conditions, composting is run under controlled conditions in a thermal insulated composting chamber. A standard mixture of raw materials is used for cultivation of the white button mushroom (*Agaricus bisporus* (J. Lange) Imbach) obtained from a local mushroom compost-producing company. Contaminated soil was mixed with the substrate and the ventilation of the composted pile inside the composting chamber followed the ventilation course of Phase 2 composting. The composting of contaminated soil was a more robust treatment than mycoremediation; however, significant differences in PAH decrease were observed among individual soils originating from different industrial sites.

Our industrial society produces and uses tremendous amounts of various synthetic polymer materials. The contemporary annual world production of synthetic polymers is around 200 million tons. Though an increasing volume of various commodity plastics is recycled in the last decades, a substantial part of plastic waste ends in dumps in the ecosystem. A typical feature of the decisive majority of synthetic polymers is their recalcitrance to any microbial attack.

Once such a material enters the natural ecosystem, the negative effect is long-lasting. That is why plastics contribute considerably to the contamination of the environment. Simultaneously with the development of technologies for physical, energetic, or chemical recycling of polymeric waste, an increasing interest is devoted to the synthesis and characterization of polymers with enhanced sensitivity to biodegradation. The employment of biodegradable polymers, namely, in applications with a short life cycle of the products, such as packaging, would be an ecologically affordable alternative for reducing solid plastic waste.

Two types of copolymers were prepared – poly(ester-amide)s by the anionic copolymerization of ϵ -caprolactam and ϵ -caprolactone, and aromatic-aliphatic copolyesters based on glycolized polyethylene terephthalate from used beverage bottles and ϵ -caprolactone. Biodegradation tests were performed by the two above-mentioned methods: composting under controlled conditions and treatment with ligninolytic fungi. Both methods resulted in degradation of the copolymers, composting being more robust. Out of fungal strains tested, *Inonotus hispidus* (Bull.: Fr.) P. Karst. degraded aromatic-aliphatic copolyesters most intensively. The results are shown on scanning electron microscopy photographs.

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Treatment of C.I. Reactive Orange 16 with the White Rot Fungus *Irpex lacteus*—Degradation Intermediates and Their Effect on Fungal Enzymes

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The capability of white rot fungi to decolorize the synthetic dyes under different culture conditions has been already well described. It is usually connected with ligninolytic enzyme activities, e.g., laccase, lig-

nin peroxidase, and manganese-dependent peroxidase, produced by the fungal cultures. However, little is known about the degradation mechanisms used by these fungi under *in vivo* conditions.

The study focuses on Reactive Orange 16 (RO16) degradation products obtained by the treatment of dye with *Irpex lacteus* cultures immobilized on polyurethane foam in a laboratory trickle-bed reactor. In these cultures 80% dye decolorization was achieved within 24 h. Dye degradation products were investigated using LC-MS analysis. Three compounds were identified as dye intermediates: 6-acetamido-3,4-dioxo-3,4-dihydronaphthalene-2-sulfonate (m/z 294), (E)-2-(4-acetamidophenyl)-1-carboxyethenesulfonate (m/z 284), and 4-(2-hydroxyethylsulfonyl)-phenolate (m/z 201). A possible pathway for RO16 degradation by *I. lacteus* was suggested. Only laccase and manganese peroxidase activities were detected in the fungal cultures suggesting these enzymes could play a role in dye decolorization. Over long periods of time, laccases are known to catalyze coupling or even the polymerization of azo dye degradation products. This fact is overlooked in many studies dealing with azo dye degradation. Despite significant laccase activities detected in the fungal cultures in this study, no backward polymerization of the reaction products resulting in recurrent colorization was observed after fungal treatment of the dye solution, making the

application of immobilized *I. lacteus* cultures more effective in azo dye decolorization than the *in vitro* enzyme treatments.

In the next part of the study, the effect of RO16 and other selected synthetic dyes on the isoenzyme pattern of ligninolytic enzymes produced by *I. lacteus* was studied. The results showed new manganese peroxidase isoenzymes produced by the fungus when grown in the presence of the dyes. Their ability to decolorize synthetic dyes was tested under *in vitro* conditions.

To summarize, previous results have indicated that the fungus *I. lacteus* has a high capacity of degradation of organic pollutants. This work brings new findings about dye degradation mechanisms used by the fungus and about the effect of synthetic dyes on the fungal cultures. Results will help in the development of fungal bioreactor cultures applicable for bioremediation technologies.

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