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SCREENING OF WHITE ROT FUNGI FOR BIOREMEDIATION OF CONTAMINATED SOIL

Principal Investigators

C G DOSORETZ
V ŠAŠEK

Grantee Institution

MIGAL-Galilee Technological Center
Institute of Microbiology, Academy of Sciences of the Czech Republic

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A.I D Grant Project Officer
Bill Crane

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EXECUTIVE SUMMARY

Environmental pollution is a global problem and the Czech Republic belongs among the countries where air, water and soil pollution are highly pronounced Biological degradation of hazardous compounds is considered to be a cost-effective and environmental friendly approach Besides bacteria, lignin-degrading fungi have been considered to be used as the degraders of aromatic organopollutants However, the practical use of these fungi is retarded by two facts (i) only few species have been studied up to now in detail, and (ii) the basic knowledge on the involvement of fungal enzymes in the degradation process is not sufficient, and the role of different physico-chemical and biological factors affecting the degradation of organopollutants under soil conditions is known only partially That is why the project started with a screening of a collection of ligninolytic fungi for their ability to produce ligninolytic enzymes, their capability to degrade selected aromatic organopollutants (PAHs, PCBs, synthetic dyes) both in liquid media and artificially spiked soil, and finally their effectiveness to remediate soil originated from polluted sites Based on the screening, a fungal strain of *Irpex lacteus* was selected as the most powerful microorganism and became a central research model in the last phase of the project Different types of biological toxicity tests were adopted to follow the toxicity changes during the bioremediation process Since a new biotechnology prospective for soil remediation – composting – emerged during the time of project study, this method was tested under field conditions, too

As a result of the project, the Czech laboratory became a recognized center of basic research of microbial soil remediation It started a cooperation with two local environmental companies and the EarthFax Development Corp , Utah, USA The Laboratory is consulted by the Czech National Health Institute on the evaluation of soil remediation technologies and have been entrusted with the organization of the NATO Advanced Research Workshop in the Czech Republic in 2000

RESEARCH OBJECTIVES

Massive amounts of xenobiotic pollutants that have been produced by the modern industry represent a serious menace to the environment Many of these toxic and often also mutagenic and cancerogenic compounds, *e g* polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), nitroaromatics, dioxins, synthetic dyes, chlorophenols and many other, persist in the environment and, especially contaminated soils and sediments can be considered a "time bomb" The number of discovered and registrered sites that require bioremediation has been growing For example, in 1998, approximately 50 000 sites in Germany were suspected to be contaminated and the US Environmental Protection Agency listed at the end of 1994, 40 000 uncontrolled hazardous waste sites and 1300 National Priorities List (Superfund) sites in North America (*sec*, May *et al*, 1997) The Czech Republic belongs to the countries with vastly polluted environment, and beside sites contaminated due to industrial and agricultural activities, also the former military bases that had been used by the Soviet troops will require a long-term clean-up

Disposal of contaminated materials has become increasingly difficult Bioremediation is thought to be a cost competitive alternative to the dumping, incineration and chemical processes that sometimes even create new ecological problems Use of microorganisms for clean-up of polluted soils holds a great promise for inexpensive and nondestructive way of remediation, making possible the decontamination of bulk volumes of contaminated material Alexander, 1994, Aust *et al* 1993) The bioremediation method has been established to exploit mostly bacterial microorganisms (Chaudhry and Chapalamadugu, 1991) and its limits have been repeatedly reviewed (Morgan and Watkinson, 1989, Providenti *et al*, 1993) The most simple (and sometimes efficient) way of reduction of pollutants in contaminated soil is the enhancement of growth and metabolic activity of autochthonous microflora by addition of mineral and/or organic nutrients, and supplying oxygen to ensure aerobic processes

Lignin degrading fungi have received considerable attention for their bioremediation potential In nature these fungi colonize lignocellulosic materials, playing a role of litter decomposers or they inhabit living or dead wood causing white rot of wood (that is why they are commonly named white rot fungi) The enzymes involved in lignin breakdown are extracellular and have a low substrate specificity These fungi have been discovered to decompose various recalcitrant organopollutants like PCBs, PAHs, DDT, dioxins, chlorophenols, nitrotoluene, azo- and polymeric dyes, different pesticides (Bumpus *et al*,1985, Eaton, 1985, Bumpus and Aust, 1986, Higson, 1991, Hammel *et al*, 1992, Joshi and Gold, 1994, Bogan and Lamar, 1995, Paszczynski and Crawford, 1995, Yadav *et al* 1995) By far most studied lignin degrading fungal species in this respect has been *Phanerochaete chrysosporium* and out of many hundreds of species possessing ligninolytic activity, only few more became research subjects (Loske *et al*, 1990, Morgan *et al*, 1991, 1993, Field *et al* 1992, 1993, Šašek *et al*, 1993, Davis *et al*, 1993, Vyas *et al* 1994a,b, Boyle, 1995, Bezalel *et al*,1996a,b, Bogan and Lamar 1996, Novotny *et al* 1997, Sack and Fritsche, 1997, Martens and Zadrazil, 1998) From the listed papers it can be concluded that the biodegradation ability of ligninolytic fungi has been proven, also in the experiments when the fungus was introduced in a contaminated soil However, the degradation was uncompleted even under ideal laboratory conditions, and the experiments with soil clean-up usually took several months

Originally the ability of ligninolytic fungi to degrade numerous recalcitrant aromatic organopollutans was attributed to ligninolytic enzymes - lignin peroxidase, manganese-dependent peroxidase and laccase, and many of those aromatics were degraded by using isolated ligninolytic enzymes (see the list summarized by Field *et al* (1993) and

references therein) However, several papers have been recently published (Thomas *et al*, 1992, Yadav and Reddy, 1993, Sack and Gunter, 1993, Yadav *et al*, 1995, Novotny *et al* 1997) that put in doubt a direct correlation between the activity of ligninolytic enzymes and xenobiotic aromatics and thus support conclusions of Field *et al* (1993) that "other, unidentified enzymes are implicated" This view has recently been supported also by Bogan *et al*, 1996) who found that fluorene oxidation in vitro was a consequence of lipid peroxidation mediated by *P chrysosporium* Mn-peroxidase Despite the fact that the role of ligninolytic enzymes in destruction of aromatic pollutants has not been elucidated completely, the ligninolytic fungi undoubtedly represent powerful prospective tool in soil bioremediation It was well demonstrated by Lamar and coworkers (Lamar *et al*, 1990, 1994) using inoculum of *P chrysosporium* and *P sordida* in field trials with soil polluted with pentachlorophenol and PAHs Polutant removal from the soil reaching up to 90% within few months seems very promising The process using these fungi has been patented (*e g* Aust *et al* 1986, Volfova *e al* 1995) and few companies included the use of ligninolytic fungi for soil remediation into their program (Intech 180, Utah, USA (Aust and Benson, 1993) or Gebruder Huber Bodenrecycling in Germany (May *et al* 1997), however, the broader application has not been put to effect yet

The use of ligninolytic fungi for soil remediation is hindered by two main reasons First, our knowledge of this process is limited, out of several thousands of white rot fungal species only one (the already mentioned *P chrysosporium)* has been studied in detail, and several more species tested only in a very incomplete way The second problem is the soil itself Soil is an extremely variable matrix, diverse in many aspects (chemical, physical, biological) and, up-to-now, no universally usable organism or a universal way of soil remediation have been described because of the influence of many environmental factors That is why, a particular procedure has to be designed for each individual contaminated site The basic six critical environmental factors controlling *in situ* biodegradation, *i e* soil water, oxygen, redox potential, pH, nutrient status and temperature (*sec*, Holden and Firestone, 1997), should be integrated with qualitative and quantitative data about the respective pollutant(s), knowledge on autochthonous soil microflora, and characteristic of the ligninolytic fungus inoculum to be used (Šašek *et al*, 1996) Proper environmental conditions are of utmost importance for growth and survival of the microorganisms involved in biodegradation This point is even more important in the case of artificially introduced microorganisms and specially stressed in the case of white rot fungi that occupy a different ecological niche in nature On the other hand some authors report that viable particles of white rot fungi can be found in soil, their amount being several orders in magnitude inferior to that of soil bacteria (Radke *et al* 1994) However, poor knowledge of capability of artificially introduced white rot fungi to grow and survive in soil exist An active fungal growth in the soil to be remedied is important in order that the pollutant would be removed in the whole volume of the soil treated Loske *et al* (1990) observed living mycelium of white rot fungi in soil several weeks after inoculation We observed the appearance of *Pleurotus ostreatus* fruit bodies in the decontaminated field where the preparation „Biodegradin" (Volfova *et al* 1995) that included straw grown mycelium of *P ostreatus* was applied

Bioremediation process normally takes place in nonsterile soil The bacterial flora present may function as a negative factor suppressing growth and development of the introduced fungus Radtke *et al* (1994) tested some pseudomonads isolated from a trinitrotoluene-polluted soil for the antagonism against growth of *P chrysosporium* on agar plates The antagonism depended on the nutrition concentration and pH The study brought evidence that the fungus was inhibited by pseudomonads producing phenazines

Another generally important factor is the availability of xenobiotics to microorganisms growing in soil The process of sorption of xenobiotics to soil particles has been recognized as

competing with biodegradation (Providenti *et al*, 1993) Soil sorption of neutral, hydrophobic compounds, such as PCBs and PAHs, is dependent on soil organic content Weissenfels *et al* (1992) demonstrated that presence of great amount of organic carbon in soil may result in a negligible biodegradation of PAHs Hydrophobic pollutant desorption may be enhanced in the presence of surfactants that increase surface areas for contact with microbial cells This effect need not be ensured by the addition of a synthetic surfactant since some of soil-borne microorganisms produce biosurfactants that are capable to enhance biodegradation of hydrophobic compounds (Oberbremer *et al*, 1990, Van Dyke *et al*, 1993) Further factors - volatilization, microbial incorporation into organic soil matter and changes in soil toxicity have to be also taken into account (Kastner *et al*, 1995, Eschenbach *et al* 1995, Kastner and Mahro, 1996, Zachař *et al*, 1996)

In the Czech Republic after political changes in late 80 s, many sites heavily contaminated with different pollutants, especially those serving as bases for the Soviet troops, were left by the previous regime It was evident that it represented a serious problem and several new environmental companies started to be involved in bioremediation technologies The rationale of the project was the necessity of serious basic research background and a search for new microorganisms that could be used in the clean up of polluted soils The innovation aspect of the project was in its complexity, covering physiology of the degrading microorganism (enzymatic activities, growth parameters, interaction with autochthonous microflora in soil), methods of extraction from polluted soil and factors influencing the recovery of the respective pollutant, and besides analytical methods of estimation, the biological toxicity test have been involved during the process of soil remediation

The research in the Czech laboratory was supported by the Institute of Microbiology since this institution covered all expenditures connected with the running of laboratories (heating, electricity, water supply, reconstruction etc) and almost all wages of both laboratory staff and PhD students

METHODS AND RESULTS

1 Materials & Methods

Microorganisms Out of 39 strains belonging to 36 different species that were isolated from fruit bodies collected in Czech forests (Šašek et al, 1998) and 30 strains belonging to 29 species from the Culture Collection of Basidiomycetes (CCBAS) of the Academy of Sciences of the Czech Republic, based on the screening on decolorization of model synthetic dyes, following strains of white rot fungi were used in the study *Bjerkandera adusta*, *Ceriporia metamorphosa*, *Daedaleopsis confragosa* *Irpex lacteus*, *Mycoacia* sp, *Phellinus pseudopunctatus* *Pleurotus ostreatus* *Stereum rugosum* and *Trametes versicolor* In some parts of the study, *Phanerochaete chrysosporium* Burds, strain ME 446 (ATCC No 34541) was used for comparison

In the study of antagonism the bacterial strains of *Pseudomonas putida*, strain DSM 8368 (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) and *Sphingomonas* sp (gift from Dept Biotechnol, University de Minho, Braga, Portugal) were included

Cultivation of fungi in liquid media. Malt extract/glucose medium (MEG), yeast extract/peptone/glucose medium (YEPG) and a low nitrogen mineral medium (Tien & Kirk,1988) containing 2 4 mM (LNMM) or 45 mM diammonium tartrate (HNMM) as the nitrogen source were used Shallow liquid stationary cultures were grown in 250-ml

Erlenmeyer flasks containing 20 ml of a medium and kept at the corresponding temperature without shaking

Nonimmersed liquid culture systems consisted of fungal mycelium immobilized on porous pieces of polyurethane foam saturated with liquid medium and highly exposed to gaseous oxygen (Dosoretz et al , 1993) Cultures were incubated with agitation in 250-ml Erlenmeyer flasks containing 90 ml of culture and 1 8 g of polyurethane foam cubes embedded in liquid medium Aeration of cultures was either by passive air diffusion through a cottonwool stopper or by daily flushing with oxygen

Cultivation of *Irpex lacteus* for enzyme isolation *Irpex lacteus* was maintained at 4°C on 2% malt extract agar slants The inoculum was prepared by transferring 5 agar plugs (1 cm diameter) of *I lacteus* grown to potato dextrose agar (PDA) into 250 ml flasks containing 90 ml potato dextrose broth (PDB) and incubated at 28°C in a rotatory incubator at 120 strokes per min After 3 days of growth the cultures were homogenized in a Waring blender for 0 5 min and used as inoculum of the experimental cultures (9 ml into 250 ml flasks containing 81 ml of a growth medium) The experimental cultures were grown (pH 4 5, 28°C) in nitrogen limited medium (56 mM glucose, 2 4 mM di-ammonium tartrate) according to Tien and Kirk [1988] in a non-immersed liquid culture system, immobilized on polyurethane foam cubes, as previously described [Dosoretz *et al*, 1993], under conditions of free air exchange To monitor the effect of manganese deficiency on LIP and MNP formation, cultures were grown in the absence of manganese All experiments were conducted with at least three replicates

Cultivation of fungi in straw and soil When enzyme production was determined in straw and soil, both components in Erlenmeyer flasks were separated by gauze Aeration was either by air diffusion through the cottonwool plug or by daily flushing of rubber-stoppered flasks with oxygen

For simultaneous estimation of the ectivities of ligninolytic enzymes and degradation of PAHs or PCBs separately in straw and soil compartment, the tube reactor system (Novotny *et al*, 1999) was used When only degradation of a pollutant in soil was studied, either mixture of straw-grown fungus with soil or sandwich layers of both were used (see Results)

Growth of fungal exploratory mycelium into soil was measured using soil columns in test-tubes inoculated with a fresh straw-grown inoculum at the top Length of the soil column colonized in time was measured to calculate the growth rate

Soil characteristics Soil Braunschweig (B) is a brown soil having pH 5 3 that contains 0 8% of total organic carbon and 0 08% (w/w) of Kjehldahl determined nitrogen Soil Prague (P) is a brown soil having pH 6 4 that contains 2 7% of total organic carbon Soil Michle (M) is a former gas works soil contaminated with PAHs (total 288 ppm) naphthalene 46, acenaphtylen 26, acenaphten 1 6, fluorene 14, phenanthrene 48, anthracene 14, fluoranthene 37, pyrene 29, benzoanthracene 14, chrysene 8 4, benzo(b)fluoranthene 9 3, benzo(k)fluoranthene 5 6, benzo(a)pyrene 13, dibenzoanthracene 1 3, benzo(ghi)perylene 11, and indenopyrene 10 ppm In addition to PAHs the soil also contains heavy metals at the following concentrations Cu^{2+} 27, Hg^{2+} 0 25, Pb^{2+} 40 and Cd^{2+} <0 5 ppm, total nonpolar extractable compounds at 1400 ppm and CN 1 2 ppm

Enzyme assays Ligninolytic enzyme activities were estimated in the stationary culture- and the nonimmersed culture liquids after the fungal biomass was removed and the filtrate desalted on a Sephadex G25 column In the straw/soil cultures, the two solid phases, straw and soil, colonized by the fungus were separated and the total amount of each of them extracted with succinate/lactate buffer After desalting on a Sephadex G25 column the enzyme activities were measured

Lignin peroxidase (LIP) activity was assayed with veratryl alcohol (Tien & Kirk, 1988), manganese-dependent peroxidase (MnP) activity was estimated either with 3-dimethylaminobenzoic acid (DMAB)/3-methyl-2-benzothiazolinone hydrazone

hydrochloride (MBTH) as the chromogen (Novotny et al , 1999) or by the method of Kuwahara et al (1984) with phenol red as the substrate Laccase was measured with 2,2-azinobis(3-ethylbenzo-thiazoline-6-sulfonic acid) (ABTS) as the substrate (Niku-Paavola et al , 1988)

One unit of enzyme activity is defined as an activity producing 1 μmol of the product per min under the assay conditions All enzyme activities were measured at the room temperature

Enzyme fractionation and heme-protein characterization A volume of 4 l of extracellular fluid was taken from cultures grown without manganese on day 12 of incubation The extracellular fluid was concentrated 40-fold by ultrafiltration, using a 10-kDa cutoff YM-10 membrane (Amicon, Danvers, MA), centrifuged for 10 min at 25,000 g, then dialyzed against 10 mM sodium acetate at pH 6 0 Samples were fractionated and analyzed for heme protein by anion-exchange HPLC, using a Mono Q column (Pharmacia, Piscataway, NJ) with a linear gradient of 10 mM to 1M sodium acetate at pH 6, at a flow rate of 1 ml/min and monitoring at 409 nm [Dosoretz *et al* , 1993] Similar procedures were applied to analyze the heme-protein composition of the culture grown with manganese except that extracellular fluid samples were taken on day 10 of growth, and a Resource Q column (Pharmacia) was used instead of Mono Q column, with flow rate of 0 8 ml/min Heme proteins were classified according to their order of elution and based on activity tests

Polyacrylamide gel electrophoresis (PAGE) and isoelectric focusing (IEF) SDS-PAGE was performed on slab gels containing 12% polyacrylamide, according to Laemmli [1972], using a Bio-Rad Mini Protean II gel electrophoresis system SDS-70 proteins kit (Sigma, St Louis, Mo) was used as molecular weight marker Proteins were stained with Coomassie Brilliant Blue G-250 Alternatively, proteins were subjected to immunological (western) blot analysis by applying rabbit antibody against LIP isozyme H1 of P chrysosporium and detecting the antibody weight goat-anti-rabbit horsereddish peroxidase The isoelectric point (pI) of LIP isozymes was determined with a Bio-Rad Mini IEF apparatus (Model 111), using broad pH range (3-10) thin-layer polyacrylamide gels A broad range Bio-Rad pI calibration kit (Catalog No 161-1010) was used as a standard and proteins were stained with Coomassie Brilliant Blue G-250 Alternatively, LIP isozymes activity was detected by activity staining in 50 mM sodium tartrate, pH 4 0 containing 0 4 mM H2O2 and 2 mM 2,6-DMP [de Jong *et al* , 1992]

Deglycosylation Asparagine-linked carbohydrate was removed from the purified LIP isozymes by digestion with recombinant N-glycosidase F (PNGase F, Biolabs, New England) After denaturing 10 μg of purified LIP isozymes by boiling it in 0 5% (w/v) SDS and 1% (v/v) β-mercaptoethanol for 10 min, the sample was added to 1% (w/v) Nonidet P-40 and 50 mM sodium acetate, pH 6 0 and incubated for 1 h with 1,000 units of recombinant PNGase F at 37°C Digestion was terminated by boiling for 5 min The sample were then subjected to SDS-PAGE analysis and proteins were stained with Commassie Brilliant Blue G-250

N-terminal sequence analysis The N-terminal sequence of LIP isozymes was determined by automated Edman degradation in an Applied Biosystems ABI494 pulsed-liquid protein sequencer (Perkin Elmer), in the National Laboratory for Biotechnology, Israel Institute of Technology, Technion, Haifa, Israel

Kinetic study of LIP isozymes The kinetic constants (K_m and k_{cat}) of the purified LIP isozymes were determined from Lineweaver-Burk plots of activity profiles at each substrate concentration Veratryl alcohol and H_2O_2 were used as substrates in the range of 100-1,000 and 20-100 μM, respectively The reactions were carried out at 25°C in sodium tartrate buffer (125 mM, pH 3 5), and absorbance changes, due to the formation of veratryl aldehyde, were followed at 310 nm [Tien and Kirk, 1988] The activity towards selected substituted phenols was measured under the same conditions

Biodegradation of PAHs In MEG stationary liquid cultures, PAH removal after 2 and 4 weeks was determined 1 week-old cultures killed by autoclaving were used as the controls PAHs were extracted from acidified medium with cyclohexane HPLC detection was employed

If degradation of PAHs in soil was studied, amounts of 6 g of tyndallized dry soil (80 °C for 6 h, for 3 consecutive days, 20% final moisture, w/w) were contaminated with 2 g of sterilized, sand spiked with PAHs (initial concentration of each PAH, 50 ppm), mixed with a freshly prepared, straw-grown fungal inoculum (8 g) in 100-ml Erlenmeyer flasks 2-week-old cultures killed by autoclaving were used as controls PAHs were extracted using supercritical fluid extraction (SUPREX PrepMaster/AccuTrap/ variflow restrictor) performed in two fractions A total sample of soil, sand and straw was extracted, first at 400 atm, 150 °C, flow rate 1 ml CO_2/min, for 10 min, then at 450 atm, 150 °C, flow rate 1 ml CO2/min using 10% (v/v) methanol as modifier, for 10 min Cryogenic collection was used glass beads, -20 °C HPLC detection was similar as mentioned above

Remediation of an actual contaminated soil (M) differed from the above described procedure in that the soil was not tyndallized, in flask the contaminated soil was sandwiched between two layers of the straw-grown fungal inoculum Apart from untreated soil the same was pretreated with a commercial soya vegetable oil

Biodegradation of dyes The following dye compounds were included in the study Methyl Red, sodium salt Methyl Orange, Reactive Orange 16, Congo Red, Reactive Black 5, Naphthol Blue Black, Remazol Brilliant Blue R, Disperse Blue 3, Fluorescein, sodium salt, Methylene Blue, Bromophenol Blue, poly R-47, and copper (II) phthalocyanine 3, 4, 4, 4-tetrasulfonic acid, tetrasodium salt

Decolorization of dyes on agar plates was evaluated using a simple technique (Novotny et al , 1997) when a mycelium-covered disc cut from an agar culture of the corresponding fungus was inserted in the center of an agar plate containing 200 ppm of the dye Control agar plates without fungal inoculation were always included in the experiments

Decolorization in stationary liquid cultures was carried out in Erlenmeyer flasks containing LNMM medium to which a dye was added at an initial concentration of 150 ppm Inoculation was with a gently homogenized liquid culture The cultures were incubated at 22 °C and, in time intervals, were sacrificed and the culture filtrate and fungal biomass were separated by decantation and centrifugation The filtrates were then diluted with the fresh medium for their absorbance values to be in the linear interval of the absorption/concentration calibration curves of the individual dyes and the absorbances were measured at the respective dye absorption maximum wavelengths (cf λmax values above) using an Eppendorf Analog Photometer 1101M (Eppendorf Geratebau, Germany) pH of the filtrates was checked before and after dilution to know the shift of pH during cultivation and to ensure measuring the dye absorbance at similar pH during the experiment The dye removal values were related to the initial concentration of the dyes 1-week old cultures killed by autoclaving were used as the control

Degradation of dye in soil was performed in 100 ml Erlenmeyer flasks with 8 g of dry and tyndallized soil that was spiked with RBBR stock solution resulting in final RBBR content of soil at 150 μg/g The spiked soil was individually treated with freshly prepared straw-grown *I lacteus* inoculum (8 0 g) and/or bacterial consortium (isolated from dye-contaminated soil in India) cultivated in the BH medium (1 1 ml of 1,63 x 10^9 cfu/ml) Respective controls were prepared similarly by adding heat-killed (autoclaved) inoculum An additional control was prepared containing contaminated soil, sterile straw and BH medium

Ecotoxicity tests The folowing three types of tests have been employed (i) Terrestrial plant growth tests were performed using water and organic solvent extracts of soil samples With each extract a filter paper was soaked and 100 seeds of different plant species were

placed over and the germination was measured after 72 hrs (ii) *Daphnia magna* acute immobilization test was carried out with 50 ml of soil water extract with 10 daphnia infants and after 24 hrs the inhibition of motility was observed (iii) Solid-phase microbial toxicity test involved addition of pH indicator dye and *Bacillus cereus* culture into the soil suspension After 2 hrs incubation the change in absorbance maxima of the dye (due to the reduction of the dye by bacteria) were measured

2. Results

Decolorization

Capability of decolorization of the dyes poly R-478 and Remazol Brilliant Blue R (RBBR) is considered to be a measure of ligninolytic activity of white rot fungi (Field et al , 1993, Pasti & Crawford, 1991) Since the ability to decolorize the former dye has also been correlated with biodegradation of some PAHs (Field et al , 1992), the ability to decolorize both dyes, of which the polymeric poly R-478 can be attacked only using extracellular enzyme activities (Field et al , 1993), was tested to compare the biodegradation potential of preselected strains of white rot fungi (cf Šašek et al, 1998) with that of *I lacteus* (Table 1) The decolorization efficiency was tested in mineral and complex media because a strain usable for practical degradation of xenobiotics should not be "dainty" to realize its biodegradation potential under a wide range of environmental conditions The content of nitrogen source, that is known to control the production of ligninolytic peroxidases in white rot fungi was from 0 002-0 003% (LNMM, MEG) to 0 07% (YEPG) The ten preselected fungal strains tested could be divided into two groups, the fungi that were capable of decolorization under broad nutritive conditions (*B adusta*, *I lacteus*, *P ostreatus*, *T versicolor*, *P punctatus*) and those that required specific conditions (*P chrysosporium*, *C metamorphosa*, *D confragosa*, *Mycoacia* sp , *S rugosum*) (Table 1) From the latter group, *P chrysosporium*, *C metamorphosa* and *S rugosum* did not decolorize the dyes in yeast extract-peptone-glucose (YEPG) medium containing a higher concentration of nitrogen used in the form of an organic nitrogen source, whereas *D confragosa* showed poor decolorization on low-nitrogen mineral medium Under suitable conditions, the efficiently degrading strains, e g *B adusta*, *I lacteus*, *T versicolor*, *P chrysosporium*, were able to completely decolorize the agar plate within not more than 10-11 days from the inoculation (Table 1)

All the strains tested grew well on both mineral and complex agar media, the most rapid growth being observed with *B adusta*, *I lacteus*, *T versicolor* and *P chrysosporium* that were able to cover the whole plate with mycelium within 3-4 days (Table 2) The capability to colonize soil was also compared since it is an important characteristic in the case the fungus is employed for soil bioremediation *I lacteus*, *P ostreatus* and *P chrysosporium* efficiently colonized the sterile soil by exploratory mycelium (Table 2) *T versicolor* was capable of only a partial colonization whereas *B adusta* did not grow into the soil at all *I lacteus* efficiently colonized also nonsterile soil, which indicated its resistance to inhibitory effects by soil microflora (not shown) The results were confirmed when inhibition of fungal growth by the soil bacteria *Pseudomonas cepacia* and *Sphingomonas* sp was tested on agar plates by the method of Radtke et al (1994) Antibiotic production by bacteria, e g phenazines, is believed to result often in such a growth inhibition However, not even the use of phenazine induction medium stimulating the production of phenazines by pseudomonads resulted in any inhibition of growth of *I lacteus*, whereas growth of *P ostreatus* and *P chrysosporium* was inhibited in the presence of the above soil-associated bacteria The optimal humidity value for soil colonization by *I lacteus* was 15-20% (w/w) (not shown) *I lacteus* was also rather resistant to toxic effects of various pollutants since we observed good colonization of a nonsterile gas works soil (M) containing a total of about 300 ppm of various

PAHs, 1 2 ppm cyanide and significant concentrations of heavy metals such as Cu^{2+} and Pb^{2+} and of a soil (P) artificially contaminated with RBBR dye at 150 ppm (not shown)

Table 1 Decolorization of agar media containing poly R-478 or RBBR dyes (each 0 02%, w/v) during growth at 28°C

Fungus	Culture medium					
	YEPG[a]		MEG[b]		LNMM[c]	
	Dye					
	Poly R-478	RBBR	Poly R-478	RBBR	Poly R-478	RBBR
Bjerkandera adusta	D (7)	D (10)	D (6)	D (10)	D (11)	D (17)
Irpex lacteus	++ (20)	D (10)	D (9)	D (7)	D (11)	D (7)
Pleurotus ostreatus	+++ (20)	D (12)	D (19)	D (10)	D (9)	D (20)
Trametes versicolor	D (6)	D (10)	D (6)	D (7)	++ (20)	D (17)
Phanerochaete chrysosporium	ND	- (20)	ND	D (6)	ND	D (6)
Ceriporia metamorphosa	- (20)	- (20)	D (8)	D (7)	D (13)	D (7)
Daedaleopsis confragosa	D (7)	ND	D (7)	D (17)	- (20)	++ (20)
Mycoacia sp	+ (20)	D (14)	+++ (20)	D (14)	++ (20)	D (14)
Phellinus pseudopunctatus	+++ (20)	D (19)	D (11)	D (14)	D (14)	D (14)
Stereum rugosum	- (20)	- (20)	D (13)	D (17)	+++ (20)	D (20)

[a]YEPG yeast extract/peptone/glucose medium, pH 4 5, [b]MEG, malt extract/glucose medium, pH 4 5, [c]LNMM, low nitrogen mineral medium (Tien & Kirk, 1988) containing 2 4 mM ammonium nitrogen as the N source Decolorizing activity decolorization of one-third (+), two-thirds of the agar plate (++), almost total decolorization (+++), total decolorization without any dye shade (D), ND, not determined

The white rot fungi tested divided into two distinct groups from the point of view of the effect of the type of medium on biomass yield In *B adusta*, *P ostreatus* and *P chrysosporium*, there was more than a 20-fold difference between the growth yields in MEG and LNMM media that have a roughly similar content of total nitrogen and less than 2-fold difference in the carbon source contents The difference in biomass yields in the two media was much lower in *I lacteus*, *C metamorphosa* and *D confragosa* (see below Table 4) A reason for such different behaviour is unknown

In order to check the biodegradation power of *I lacteus*, its ability to degrade a range of synthetic dyes, monoazo-, diazo-, anthraquinone-based-, heterocyclic-, triphenyl methane- and phthalocyanine dyes, was tested in both agar media and shallow liquid cultures (Table 3) In a preliminary test, decolorization of 11 different dyes (each at 200 ppm) was tested on the complex MEG solid medium (Table 3), where the most rapid decolorization of poly R-478 and RBBR dyes was observed (cf Table 1) All types of the dyes used were completely decolorized, the slowest decolorization process taking about 20-25 days was observed with Congo Red and Naphtol Blue Black (diazo dyes) and with Methylene Blue (thiazine dye) (Table 3) In shallow liquid cultures a mineral medium was used instead of MEG medium employed in the preliminary test to avoid absorbance by the yellowish MEG medium that could interfere with spectrophotometrical estimation of dye degradation The relative

decolorization values related to the original dye concentration of 150 ppm, that were obtained in stationary liquid cultures with selected dyes after a 2-week degradation, were the following Methyl Red (monoazo dye) 56%, Congo Red (diazo dye) 58%, RBBR (anthraquinone-based dye) 93%, Cu-phthalocyanine (phthalocyanine type) 98%, and Bromophenol Blue (triphenyl methane type) >99% (Table 3) Effect of the presence of dyes on growth was also measured Growth yields in shallow liquid cultures containing the above dyes were reduced by 36-49%, the most toxic being Congo Red, Bromophenol Blue and RBBR (not shown)

Table 2 Growth of fungal strains in agar media and in sterile soil at 28°C

Fungus	Culture medium		
	MEG[a]	LNMM[b]	Sterile soil (cm/day)[d]
Bjerkandera adusta	3	4	0 (-)
Irpex lacteus	3	3	0 39 (T)
Pleurotus ostreatus	3	6	0 31 (T)
Trametes versicolor	3	4	0 22 (P)
Phanerochaete chrysosporium	3	3	0 53 (T)
Ceriporia metamorphosa	4	4	ND
Daedaleopsis confragosa	5	6 [c]	ND
Mycoacia sp	5	13	ND
Phellinus pseudopunctatus	9	8	ND
Stereum rugosum	9	22	ND

[a]MEG, malt extract/glucose medium, pH 4 5, [b]LNMM, low nitrogen mineral medium (Tien & Kirk 1988) containing 2 4 mM ammonium nitrogen as the N source, ND, not determined Growth on MEG and LNMM was estimated on agar plates, figures refer to numbers of days necessary for covering the whole surface of a Petri dish with mycelium [c]Mycelium covered only two-thirds of the plate surface and stopped growing [d]Growth measured as a length of soil column (1 5 cm diameter) colonized by exploratory mycelium of the fungus growing from a 2-cm layer of fresh straw-grown inoculum Maximal length of soil column colonizable by the fungus was 6 cm Growth rate was expressed as a mean length (cm) of soil column colonized per day by exploratory mycelium within 9-d period T, fungus was able to colonize the whole length of the soil column, P fungus colonized only a part of the soil column s length, -, no growth of exploratory mycelium in the soil

Table 3 Decolorization of synthetic dyes by *Irpex lacteus* in malt extract/glucose agar medium and in shallow stationary cultures growing in low nitrogen mineral medium at 22°C

Dye	Type	Decolorization efficiency	
		Complete decolor [1] (days)	Dye removed (%)[2]
Methyl Red	Monoazo	9	56
Methyl Orange	Monoazo	9	ND
Reactive Orange 16	Monoazo	10	ND
Congo Red	Diazo	20	58
Reactive Black 5	Diazo	11	ND
Naphtol Blue Black	Diazo	27	ND
RBBR	Anthraquinone-based	10	93
Disperse Blue 3	Anthraquinone-based	17	ND
Fluorescein	Heterocyclic	15	ND
Methylene Blue	Heterocyclic (thiazine)	22	ND
Bromo Phenol Blue	Triphenyl methane	12	>99
Cu- phthalocyanine	Metal complex Cu(II)	ND	98

[1]Figures indicate a period from inoculation (in days) necessary to completely decolorize the agar plate containing MEG medium with a dye at 200 ppm [2]Figures refer to the portion of the original dye concentration of 150 ppm removed by the fungus from the low nitrogen mineral medium (LNMM) during 2 weeks ND not determined

Removal of the dye from contaminated soil

Soil contaminated with the synthetic dye Remazol Brilliant Blue R (RBBR) was treated independently with wheat straw-grown white rot fungus *Irpex lacteus*, a bacterial consortium isolated from a dye-polluted soil and a coculture comprising both *I lacteus* plus the bacterial consortium (Fig 1)

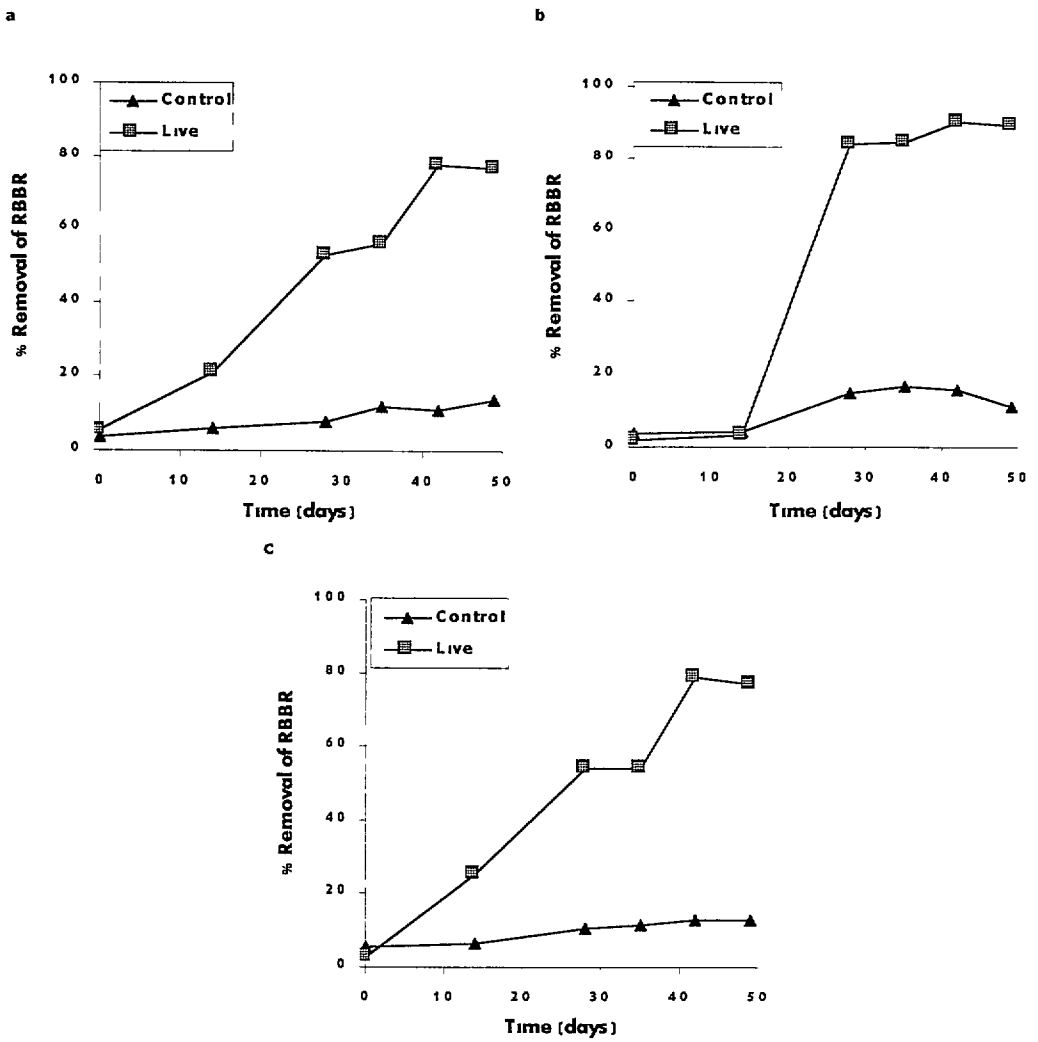

Figure 1 Removal of RBBR from contaminated soil by *I lacteus* (a), bacterial consortium (b) and a coculture of the fungus and the bacteria (c)

Both *I lacteus* and the coculture removed RBBR (decrease in absorbency at 578 nm) gradually during the 49 day incubation time to 76 and 78%, respectively The bacterial consortium alone, however, decolourised RBBR starting after 14 days with a final RBBR removal of 89% Using controls with heat-killed inoculum of the three bioremediating systems almost no decolourisation occurred The decoloration by coculture did not show an increased RBBR removal as compared to the individual cultures This might be explained by the observation that *I lacteus* inhibited growth of the bacterial consortium

Ligninolytic enzymes

Ligninolytic enzymes have been shown to be responsible for biodegradation of various recalcitrant pollutants, e g PAHs, pesticides, synthetic dyes (cf reviews Cerniglia, 1992, Hammel, 1995, Field et al , 1993, Paszczynski & Crawford, 1995, etc) and thus their

synthesis and regulation in *I lacteus* were studied under various conditions Compared to other white rot fungi efficiently decolorizing poly R-478 and RBBR, *I lacteus* was found to be able to produce a high level of MnP whose production, however, was very much dependent on the type of medium, and a medium level of laccase which was rather independent of the medium used (Table 4) As in *D confragosa*, the ligninolytic enzyme productions in *I lacteus* were significantly higher in the complex MEG medium, compared to the mineral medium (LNMM), even though the total nitrogen concentrations in the two media were not much different (approximate respective estimations based on the information on malt extract given by the producer are 0 02 and 0 03 g N/l) In contrast LIP was not present on either medium (Table 4)

Table 4 Ligninolytic enzyme activities of white rot fungi exhibiting high capacity of decolorization of poly R-478 and RBBR dyes growing in shallow liquid cultures at 28°C

Fungus	Dry weight biomass (g/L)		Enzyme activity (U/L)					
			LIP		MnP		Laccase	
	LNMM	MEG	LNMM	MEG	LNMM	MEG	LNMM	MEG
B adusta	2 2	74 4	0	0	63 3	9 5	0 3	0 7
I lacteus	8 2	12 7	0	0	0 5	177 0	3 2	5 8
P ostreatus	2 2	78 8	0	0	0 5	13 6	61 4	12 3
T versicolor	ND	ND	0	ND	23 0	ND	5 0	ND
P chrysosporium	3 8	80 0	0	1 0	2 0	4 0	0 3	0
C metamorphosa	18 9	20 1	0	0	0 7	1 4	1 9	0 2
D confragosa	10 3	14 4	0	0 6	0	15 4	16 0	47 3

Figures represent maximal enzyme activities and growth yields obtained during a 3-week cultivation
LiP, lignin peroxidase, MnP, Mn-dependent peroxidase, LNMM, low nitrogen mineral medium (Tien & Kirk, 1988) containing 2 4 mM ammonium nitrogen as the N source MEG, malt extract/glucose medium, pH 4 5, ND, not determined

A 20-fold increase in the ammonium nitrogen in the mineral medium resulted in a considerable increase of the production of MnP nad laccase, accompanied by the appearance of LIP activity (Table 5) *I lacteus* is thus able to synthetize simultaneously all three major extracellular ligninolytic enzyme activities, LIP, MnP and laccase, which is rather an infrequent situation in white rot fungi (Hattakka, 1994) In addition to those activities, extracellular pyranose-2-oxidase and RBBR oxidase activities (not shown), the latter being discovered only recently in *P ostreatus* (Vyas & Molitoris, 1996), were also detected in the extracellular fluid of shallow liquid cultures grown on the mineral medium of Tien and Kirk Immobilization of *I lacteus* in the nonimmersed liquid culture, that may partially mimic natural growth of the fungus on lignocellulosic substrates and in *P chrysosporium* led to overproduction of LIP at excess nitrogen levels (Dosoretz et al , 1993), resulted in decreased levels of ligninolytic enzymes, compared to the shallow stationary culture (Table 5)

The enzymes were also synthesized during growth of *I lacteus* on straw and in soil, with the exception of MnP whose activity was not detected in the straw and soil cultures The activities of LIP and laccase extractable from the soil colonized by exploratory mycelium of the fungus represented one tenth of the activities extracted from the straw cultures (Table 5) Aeration with pure oxygen, compared to simple air diffusion, reduced the production of LIP and MnP while the synthesis of laccase was not influenced by the intensity of aeration

Table 5 Effect of cultivation conditions on the production of ligninolytic enzymes in *Irpex lacteus*

Enzyme	Type of culture (medium)						
	Stationary (HNMM) U/L	Nonimm (HNMM) U/L		Straw U/g dry wt straw		Soil U/g dry wt soil	
	AIR	AIR	OXYGEN	AIR	OXYGEN	AIR	OXYGEN
LIP	70 (18)	0	0	6 (13)	3 4 (20)	0 4 (20)	0 3 (28)
MnP	970 (18)	831 (10)	527 (10)	0	0	0	0
Laccase	36 (18)	23 (20)	26 (10)	2 (20)	2 8 (20)	0 3 (20)	0 2 (24)

Figures represent maximal enzyme activities obtained during a 20-day cultivation on liquid media (stationary shallow liquid culture, nonimmersed liquid culture) and during a 30-day cultivation on straw or in soil (exploratory mycelium) All cultivation were carried out at 28°C Numbers in parentheses indicate the day of cultivation when the maximal enzyme activity was detected

HNMM, high nitrogen mineral medium according to Tien & Kirk (1988) containing 45 mM ammonium nitrogen as the N source, LIP, lignin peroxidase, MnP, Mn-dependent peroxidase, ND, not determined

Ligninolytic enzyme formation by I lacteus

A time course study of the ligninolytic enzyme formation by *I lacteus* was conducted in a non-immersed liquid culture system under nitrogen limitation conditions Under these conditions the three ligninolytic enzymes, LAC, MNP and LIP were detected (Fig 2) No other ligninolytic enzymes were detected The formation of the ligninolytic enzymes was obtained in a temporal sequential mode, with the peak level of LAC activity preceding that of MNP and LIP (days 4, 7, and 11, respectively) Analysis of the heme protein composition in the extracellular fluid of *I lacteus* taken on day 10, using strong anion exchange HPLC and activity tests revealed the presence of four MNP and three LIP isozymes (Fig 3) The isozymes were denominated MNP1-MNP4 and LIP1-LIP3 according to the order of elution

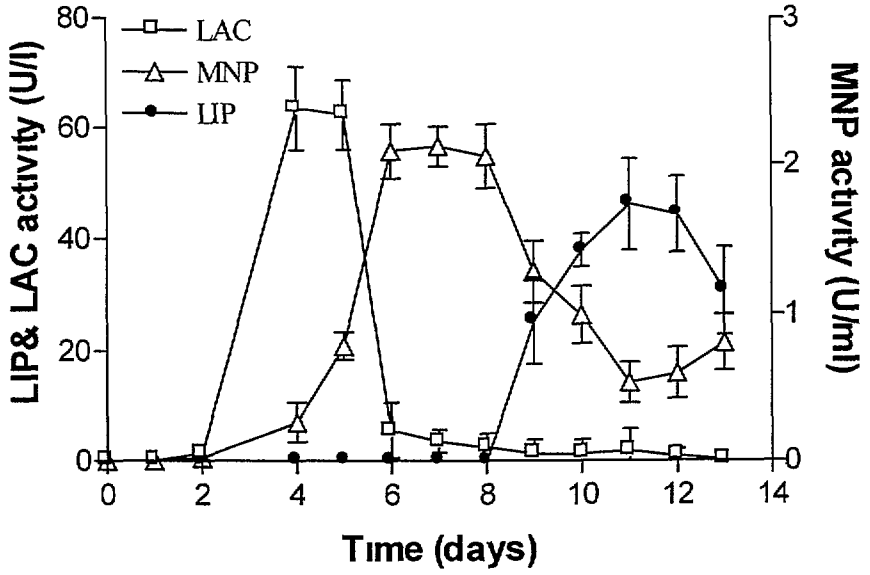

Fig 2 Time course of ligninolytic enzymes activities in the extracellular fluid of *I lacteus* cultivated in the non-immersed liquid culture system under nitrogen limitation condition Bars indicate standard error

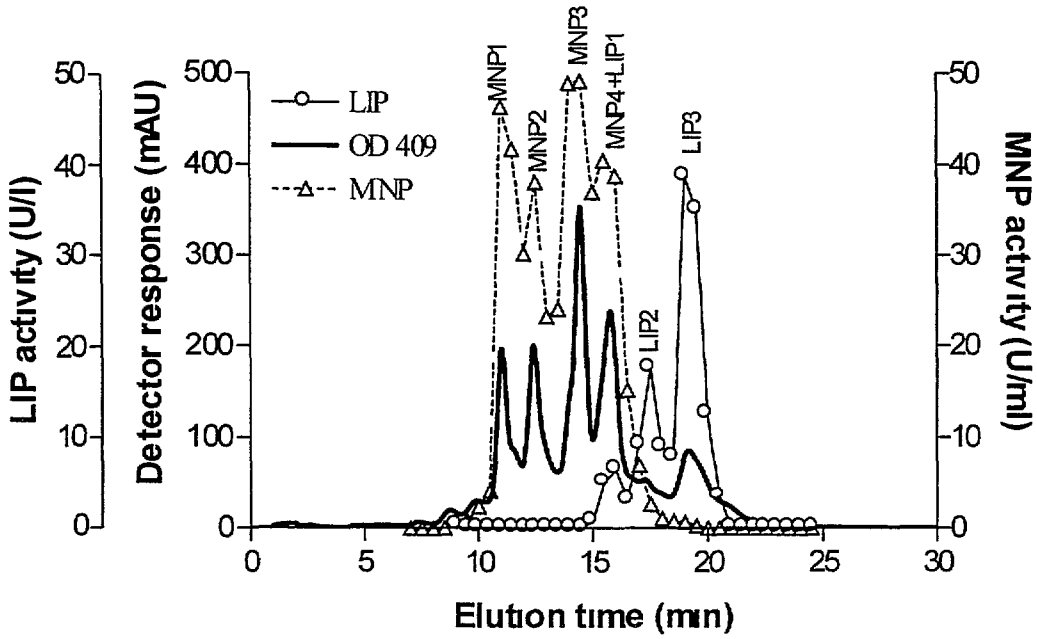

Fig 3 Anion exchange-HPLC fractionation of the extracellular fluid of 10 day old cultures of *I lacteus* grown in low nitrogen medium under air in the non-immersed liquid culture system HPLC was performed on a Resource Q column at a a flow rate of 0 8 ml/min with a linear gradient of sodium acetate (10-1000 mM) at pH 6 and monitoring at 409 nm

For the purification of LIP isozymes, *I lacteus* was cultivated in manganese-free medium in order to repress the formation of MNP As evidenced from the heme protein profile obtained for extracellular fluid of day 12 of growth, using strong anion exchange-HPLC (Fig 4), the three LIP isozyme were the dominant heme proteins whereas no MNP activity was detected In addition, an unknown heme peak denominated A, lacking LIP, MNP and MIP activity, was also oberved The specific activities obtained for the purified isozymes LIP1-3 were 78 2, 80, and 120 U/mg and the RZ factor 2 0, 2 5, and 2 5, respectively

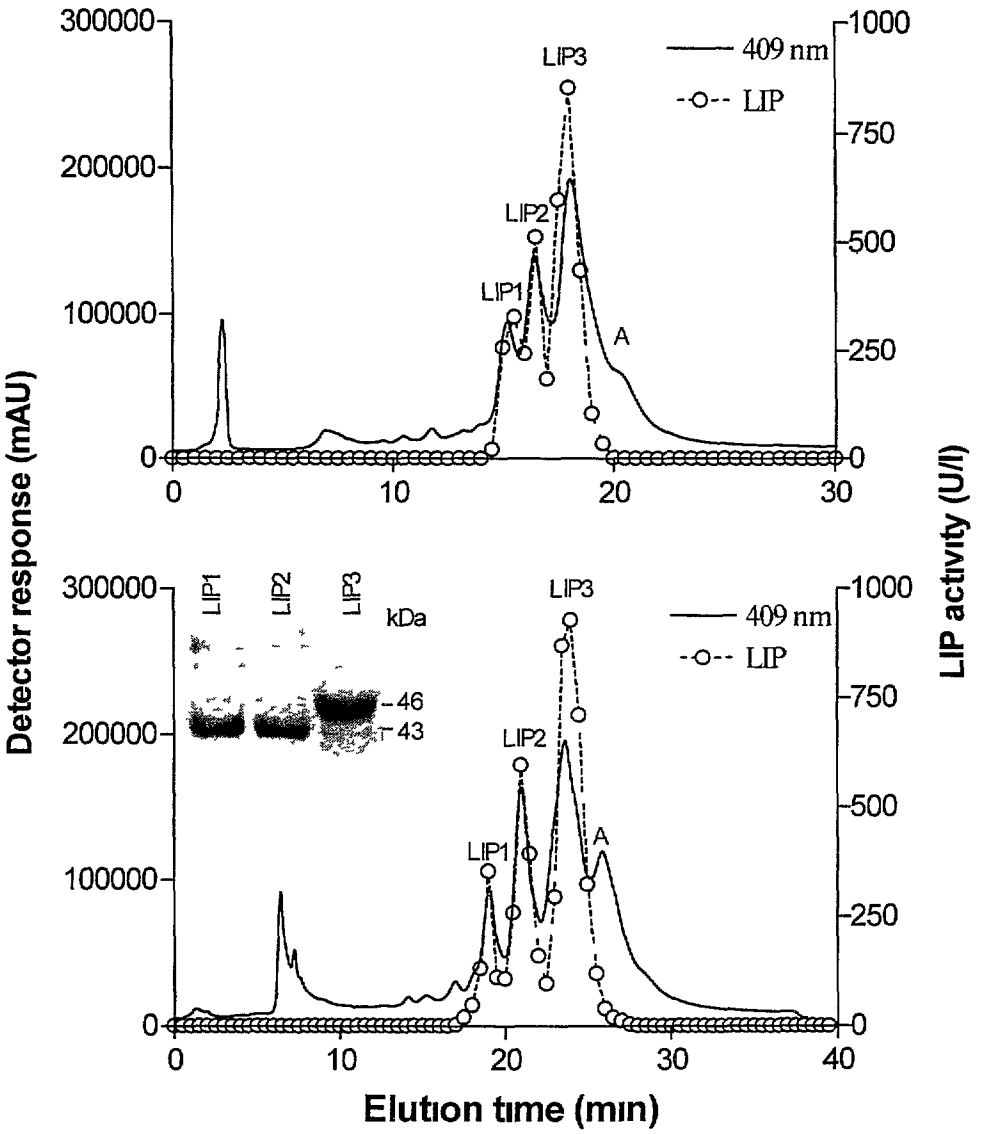

Fig 4 Anion exchange-HPLC fractionation of the extracellular fluid of 12 day old cultures of *I lacteus* grown in manganese free-low nitrogen medium under air in the non-immersed liquid culture system HPLC was performed with a linear gradient of sodium acetate (10-1000 mM) at pH 6 and monitoring at 409 nm on a Resource Q column (top), at a a flow rate of 0 8 ml/min, or on a Mono Q column (bottom), at a flow rate of 1 ml/min Insert displays SDS-PAGE of the purified LIP isozymes Protein staining was done with Coomasie Brilliant Blue G-250

The physical properties of the isolated isozymes were determined (Table 6) The molecular weights of LIP1-LIP3 determined by SDS-PAGE were found to be 41, 41, and 44 kDa, respectively Each lane resulted in only one band for each isozyme, indicating a high degree of purification Removal of the N-linked carbohydrate of the purified enzymes with N-glucosidase resulted for each single protein in a molecular mass of 39 3 kDa for LIP1 and LIP2 and 41 8 kDa for LIP3, indicating a carbohydrate content of 4-5% by weight, which is in agreement with that of other fungal peroxidases The pI values of the three isozymes (LIP1-LIP3) were estimated by gel IEF and found to be 5 0, 4 9, and <4 6, respectively Only one band of activity was obtained for each of the individual isozymes after staining the gel for peroxidase activity with DMP

The optical absorption spectra for the LIP3 isozyme was determined The absorption spectra of the native LIP3 exhibited a Soret maximum at 407 nm and visible bands at 503 nm and 634 nm (Fig 5) Addition of 40 equivalent (120 μM) of H_2O_2 to native LIP3, led to the formation of compound III, with a Soret maximum at 413 nm and a visible bands at 545 nm The absorption spectra of a LIP-cyanide complex exhibited a Soret maximum at 360 and 422 nm and a visible band at 530 nm Similar results were obtained for the other LIP isozymes of *I lacteus* The absorption maximum obtained for either native LIP, H_2O_2-oxidized LIP and LIP-cyanide complex resulted very similar to those obtained for LIP of *P chrysosporium* [Warishi and Gold, 1990]

Table 6 Physical properties of lignin peroxidase isozymes from *I lacteus*

Isozyme	Mr (KDa)[a]	pI[b]	Glycosylation[c](%)
LIP1	41	5 0	4 2 (39 3)
LIP2	41	4 9	4 2 (39 3)
LIP3	44	<4 6	4 9 (41 8)

[a]Molecular weight (Mr) was determined by SDS-PAGE and protein staining with Coomassie Brilliant Blue G-250 [b]Isoelectric point (pI) was determined by IEF gel electrophoresis and protein staining with either with Coomassie Brilliant Blue G-250 or bv activity staining with DMP [c]Degree of glycosylation was determined by removal of the N-linked carbohydrate of the purified enzymes with N-glucosidase followed by SDS-PAGE Numbers in brackets represent the Mr in kDa upon deglycosylation

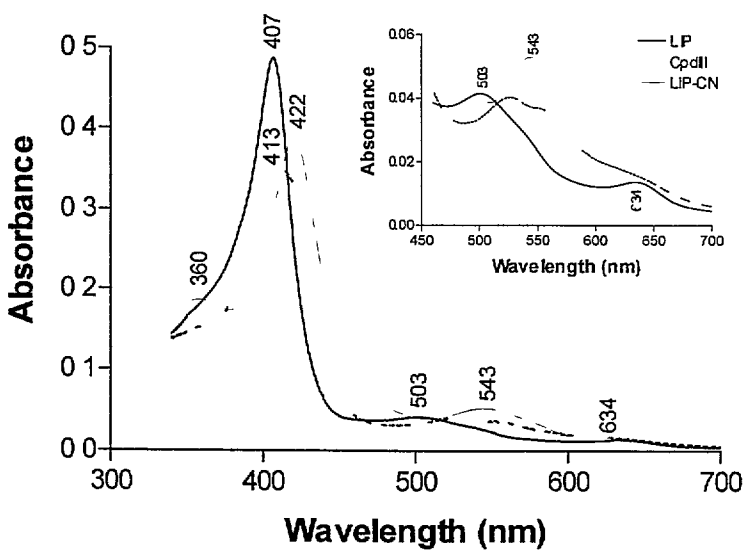

Fig 5 Optical absorption spectra of LIP3 isozyme from *I lacteus* Compound III was obtained by the addition of 40 equivalents of H2O2 to native LIP The LIP-cyanide complex was obtained by the addition of 5 mM potassium cyanide to native LIP In all cases, LIP concentration was 3 μM in 100 mM acetate buffer at pH 6 Insert shows a magnification of the visible range of the spectrum

The N-terminal amino acid sequence of the three LIP isozyme of *I lacteus* was determined (Table 7) As can be observed by comparing the N-terminal amino acid sequence of the LIP isozymes from *I lacteus* with those reported for LIP isozymes of other white-rot fungi, a low homology is evident

Table 7 Comparison of the amino acid N-terminal sequence of LIP isozymes from *I lacteus* with those reported for selected LIP isozymes produced by other white-rot fungi

LIP isozyme	N-terminal sequence
I L - LIP1	V-N-P-G-G-G-R-F-V-K-N-A-A-A[a]
I L -LIP2	V-N-P-G-G-G-R-F-V-K-N-A-A-A[a]
I L - LIP3	V- ?-?-G- D-G-R-T-V-K-N-A-A[a]
P C -H2	V-A-C-P-D-G-V-H-T-A-S-N-A-A-C-C-A[b]
B A -LPO-2	V-A-C-P-D-G-K-N-T-A-I-N-A[c]
T V -LP12	V-A-C-P-D-G-V-H-T-A-T-N-A-A-C-C-Q[d]

[a]This work
[b]Adapted from Reddy]
[c]Adapted from Kimura, *at al*
[d]Adapted from Johansson *at al*
Abbreviations I L , *Irpex lacteus* P L , *P chrysosporium* B A , *Bjrkandera adusta* T V , *Trametes versicolor*

The pH dependence in the oxidation of veratryl alcohol by LIP3 isozyme was studied (Fig 6) The highest rate of veratryl alcohol oxidation was observed at around pH 2 2 whereas only negligible activity was measured at pH 4 5 and above

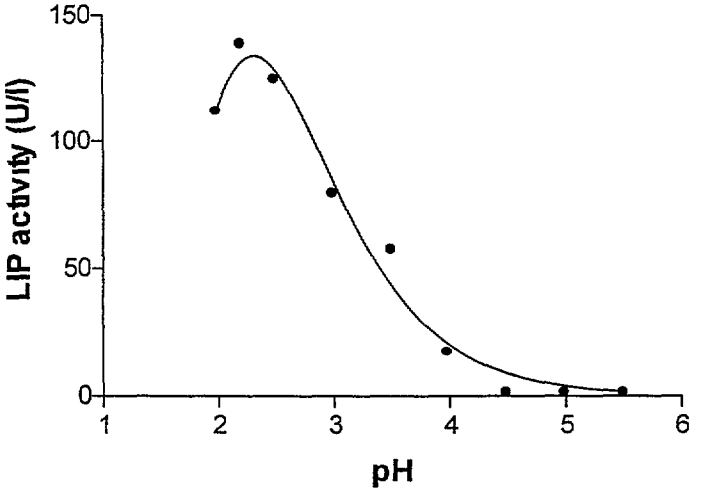

Fig 6 pH-activity profile of LIP3 isozyme from *I lacteus* Assays were done at 25°C conditions in 125 mM sodium tartrate buffer

The kinetic parameters of LIP isozymes was determined at pH 3 5 and 25°C with H_2O_2 and veratryl alcohol as substrates (Table 8) The catalytic properties of LIP isozymes, as expressed by k_{cat} and K_m values, were similar among them, and in the same range of those previously published for LIP isozymes from *P chrysosporium* strains BKM-F and INA-12 [Farrell *et al*, 1989, Glumoff *et al*, 1990, Rothschild *et al*, 1997] Like most fungal peroxidases, LIP isozymes from *I lacteus* oxidized veratryl alcohol and a variety of substituted phenols tested in the reaction, displaying, in general, a preferential reactivity for

veratryl alcohol as the electron donor (Table 9) The reactivity towards the different substrates were similar among the three isozymes Guaicol and 2,6-dimetoxhyphenol which were reported as efficient substrates for LIP from *P chrysosporium* and *B boss55*, resulted much less reactive toward LIP isozymes from *I lacteus*

Table 8 Catalytic properties of purified LIP isozymes from *I Lacteus*[a]

Isozyme	K_m-VA (μM)	K_m - H_2O_2 (μM)	k_{cat} (s^{-1})
LIP1	326 4 ± 4 5	16 6 ± 0 3	17 8 ± 2 6
LIP2	233 8 ± 6 1	15 2 ± 0 2	14 9 ± 3 9
LIP3	315 5 ± 8 3	13 6 ± 0 1	20 9 ± 6 2

[a]Kinetic parameters were determined from initial velocities LIP isozymes were incubated at 25°C in sodium tartrate buffer (125 mM, pH 3 5) K_m Michaelis- Menten constant, k_{cat} turnover number per second, VA veratryl alcohol

Table 9 Relative activity of lignin peroxidase isozymes of *I lacteus* on selected substituted phenols

Substrate[a]	λ^b (nm)	ε^c (mM^{-1} cm^{-1})	Relative activity[d] (%)		
			LIP1	LIP2	LIP3
Veratryl alcohol	310	3900	100	100	100
2,6-Dimethoxyphenol	468	27500	45	52	41
Hydroquinone	247	21028	77	87	64
Guaiacol	465	12100	19	22	18

[a]Substrate concentration was 1 mM, pH was 3 5 and temperature 25°C in all cases [b]Wavelength for maximum absorbance of the product/substrate of oxidation at which the course of the reaction was followed in each case [c]Extintion coefficient of the product/substrate of oxidation by which the course of the reaction was followed in each case [d]Relative activity is the percentage of activity for each substrate in respect to veratryl alcohol

PAH degradation

The ability of *I lacteus* to remove three- and four-ringed PAHs from the complex MEG medium was compared with those of *B adusta*, *P ostreatus*, *T versicolor* and *P chrysosporium* All PAHs tested were present simultaneously in shallow liquid cultures each at a concentration of 25 ppm The removal rates by *I lacteus* of all PAH tested, both after 2 and 4 weeks, were superior to those by other fungi The degradation of anthracene was accompanied by an accumulation of anthraquinone, much more massive than in other fungal organisms tested (Fig 7) In the case of fluorene, the removal rate by *I lacteus* was comparable to the rate observed with *P ostreatus*, but was slightly lower than the rates measured with *B adusta*, *T versicolor* and *P chrysosporium* The evaluation of phenanthrene removal was complicated due to a massive disappearance of the compound in heat-killed control systems suggests losses by volatilization or irreversible sorption that obscured a possible weak degradation of phenanthrene since the ability of *P ostreatus* and *T versicolor* to remove and metabolize phenanthrene has been rather well documented (cf Sack & Gunther *et al*, 1997, Bezalel et al, 1996b)

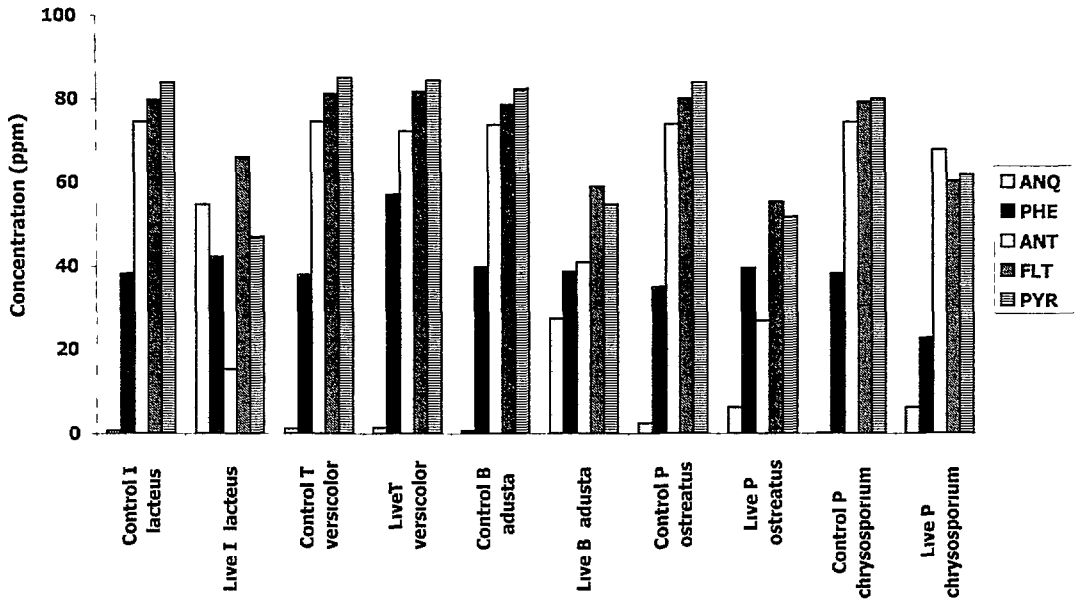

Figure 7 Degradation of PAHs in liquid medium by selected white rot fungi

The capability of removal of the above PAHs by the action of *I lacteus* was also tested in artificially contaminated soil at 50 ppm The removal rates by the fungus after 3 months for anthracene, fluoranthene and pyrene were 49, 25 and 52% of the original amount, respectively (Table 10) In this case, however, no anthraquinone accumulated Compared to the removal from liquid medium, also a net removal by the fungal culture of 37% of the spiked amount of phenanthrene was observed (Table 10) Addition of cultures of *Pseudomonas putida* and *Sphingomonas* sp did not have any effect, the bacteria did not degrade the PAHs and did not contribute to degradation of PAHs by *I lacteus* (Fig 8)

Table 10 Removal of PAHs from artificially spiked soil by *Irpex lacteus* at 26 °C during 3 months

	Removal of PAH (%)[1] ± S D (%)[2]				
	Phenanthr	Anthracene	(Anthraq[3])	Fluoranthene	Pyrene
Total removal	75 ± 10	98 ± 32	(6 ± 19)	61 ± 8	89 ± 23
Heat-killed control	38 ± 4	49 ± 8	(6 ± 4)	36 ± 7	37 ± 4
Removal by fungus	37	49	-	25	52

[1]Per cent of the original amount of PAH used for contamination (400 µg/6 g soil) = 100 % [2]Relative stabdard deviation [3]Per cent of the original amount of anthracene used for contamination Experimental conditions 6 g soil (D W) + 2 g sand + 4 g straw in 100-ml Erlenmeyers, inoculation with the fungus grown on straw, soil humidity 20 % (w/w), temperature 26 °C, aeration by air diffusion, 5 replicates, duration 3 months

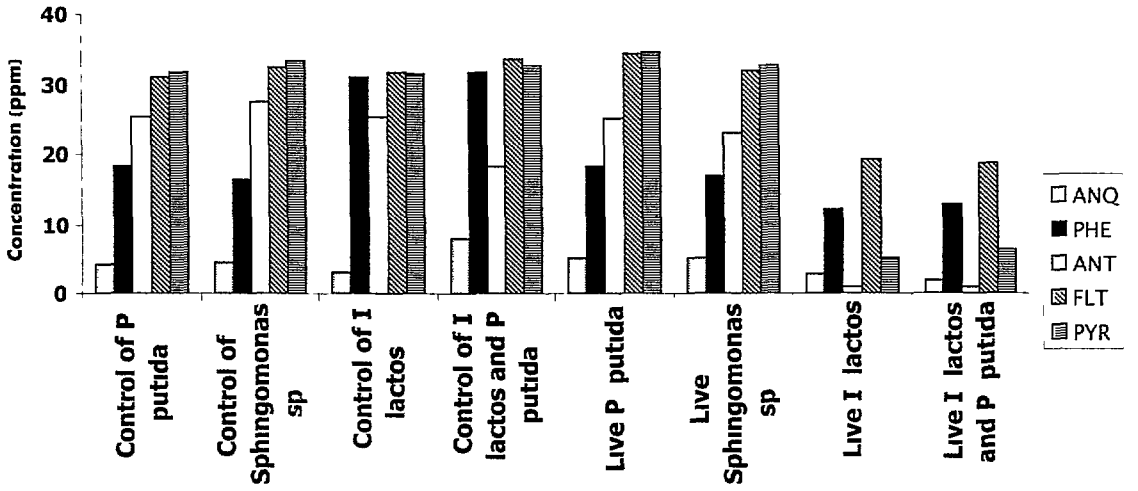

Figure 8 Degradation of PAHs in artificially contaminated soil

The PAH-degradation efficiency of *I lacteus*, both individually and in combination with *P putida*, in an actual PAH-contaminated soil (soil M - see Materials and Methods) was analysed after 5 weeks of incubation period The soil was contaminated with a number of PAHs with concentration range between 20 to 150 ppm At the end of 5 weeks of incubation *I lacteus* and the coculture significantly degraded phenanthrene, anthracene, pyrene and fluoranthene (10 to 60%) The decrease in the concentration of PAHs was not observed where individual bacterial culture was applied The current experiment is still running and the final analysis will be completed after 3 months

In the same experiment, ecotoxicity tests of soil samples, both before and after remediation, were carried out Both heat-killed control and untreated soil samples were toxic to mustard (*Brassica* sp) seeds (100% inhibition of seed germination) and *Daphnia magna* (100% mortality rate), while the soil samples treated with *I lacteus* and the coculture were found to be significantly less toxic (< 30% inhibition of seed germination and 0% mortality rate respectively) At the termination of the experiment the solid-phase microbial toxicity test has been be included

Experiments dealing with the study of biodegradation, volatilization and sorption of PCBs and PAHs using tube-reactor system are not included in this report being detailed described in the Annual Report for 1997

Field experiment - composting and application of white rot fungi.

Soil used in this experiment originated from a former accidentally contaminated site due to an oil spillage Since the content of PAHs was low, the concentration was increased through artificial spiking A pile of 500 kg of soil screened through a sieve of 0 8 mash was contaminated with anthracene, phenanthrene and pyrene in a following way 10 kg of dry, river sand (grain size < 2 mm > 0 5 mm) was gradually sprayed with a 4 5 litre of acetone, in which all above anthracene, phenanthrene and pyrene (25 g each) were dissolved The contaminated sand was transferred to decontamination field and gradually mixed with 500 kg of soil using a small excavator The final concentration of each PAH in soil was 50 ppm *Composting* 450 kg of contaminated soil was mixed (using an excavator) with 2000 kg of a mixture of raw materials obtained from a mushroom compost producing company (so called "green substrate") During the second mixing, water (300 litre) was continuously added till optimum compost humidity At the end, a pile about 2 m high was made and covered with a perforated black plastic foil to allow aeration but keep high temperature and humidity In week intervals the pile was spread out over the decontamination place, evaporated water

added and the pile was rebuild again This way of composting corresponds to a prolonged "phase I" of the fermentation of mushroom substrate The fermentation process possessed typical feature of a proper composting - temperature course inside the pile (first four weeks 65°C, next 3 weeks gradually ceasing down), changes in structure and color of straw particles (yellow straw turns dark brownish and was partially decomposed), significant loss of organic matter and continuous decrease of manure smell The concentration of all three PAHs gradually decreased throughout the composting and at the end of the process it represented only about 10% of original amount, in the 2nd week of the treatment a significant accumulation of anthraquinone was detected as a result of anthracene metabolism (Fig 9)

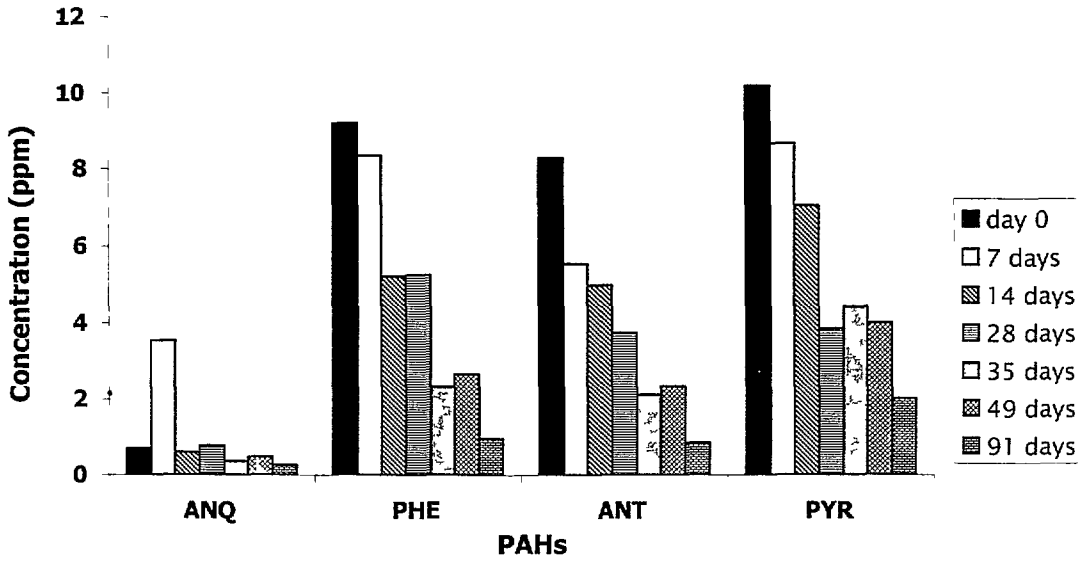

Figure 9 Composting of soil contaminated with PAHs

Application of white rot fungi 20 kg of above described contaminated soil was mixed with 20 kg of straw grown white rot fungus A layer 20-25 cm was made inside a wooden frame, the surface moistened and covered with a white, perforated plastic foil The experiment was checked in week intervals, if necessary the surface was moistened but not mixed Growth of fungal mycelium was observed visually With *Pleurotus ostreatus* fruit bodies started to form after 6 week treatment demonstrating good physiological state of the fungus However, the removal of PAHs was not found significant because the experiment was influenced by the disappearance of the polyaromatics both from the treatment fields and control soil material probably due to some physico-chemical factors That is why the experiment was repeated under green house conditions with *Irpex lacteus* Within 7-week-treatment the removal of all the 3 PAHs was in the range of 60-70%, and the significant anthraquinone accumulation was observed during the first week of the experiment

IMPACT, RELEVANCE AND TECHNOLOGY TRANSFER

Impact of the project on the research and realization of bioremediation in the Czech Republic has been diversified Basic research results of the project will influence the evaluation of the effectiveness of individual soil remediation treatments by describing all necessary steps that should precede the field trials The finding of volatilization of some PAHs PCB congeners demonstrated necessity of health protection regulation both under laboratory and field experiments

Practical impact of the project was the selection of a white rot fungus *Irpex lacteus* whose physiology and biochemical properties have been only marginally studied It has been reported to be an efficient source of extracellular cellulase-, xylanase-, D-galactanase-, and acid RNase- activities (Kanda et al , 1985,1989, Tsumuraya et al , 1990, Watanabe et al , 1995) and tested as a lignin degrader (Capelari & Zadražil, 1997) The biodegradation abilities of *I lacteus* are practically unknown since only brief information has been published so far documenting the removal of PCP from artificially-contaminated soil and its binding into humic materials by the biochemical activity of the fungus (Leštan & Lamar, 1996, Ruttimann-Johnson & Lamar, 1997) Our growth experiments characterize *I lacteus* as a robust ligninolytic fungus capable of good growth on both synthetic media and in soil, its capability of efficient colonization of nonsterile soil, where competition with autochthonous soil microflora is occurring, which is important from the point of view of possible applicability to soil remediation, when the growth of fungus in the whole volume of soil is a prerequisite of quantitative removal of the xenobiotic

During the course of the project using analytical equipment purchased from the grant, one PhD and one MSc student realized their dissertations and two PhD student started their research Students and research workers of the Czech laboratory got a training in Israeli laboratory and participate in top scientific meetings As a result of the project, the Czech laboratory became a recognized center of basic research on microbial soil remediation It started a cooperation with two local environmental companies and the EarthFax Development Corp , Utah, USA The purpose of collaboration with Czech companies is the scale-up of the remediation of PAH polluted soils, the goal of collaboration with the ErthFax Development is introduction of fungal technology into Czech Republic The Laboratory is consulted by the Czech National Health Institute on the evaluation of soil remediation technologies and have been entrusted with the organization of the NATO Advanced Research Workshop in the Czech Republic in 2000 In connection with the project, an international cooperation with the University of Regensburg, Germany, and the University of Grenoble, France has developed, both supported by the Ministry of Education of the Czech Republic and its corresponding partners in Germany and France, respectively Duration of both projects is 3 years, first project deals with decolorization of synthetic dyes, the second with PAH degradation

PROJECT ACTIVITIES/OUTPUTS

1 Meetings attended and held for entire project

a) 8th IUMS Congress, Jerusalem, Israel, August 1996
b) Mini-Symposium on Biosorption and Microbial Degradation V, Prague, Czech Republic December 1997
c) In Situ and On-Site Bioremediation, The Fourth International Symposium, New Orleans, USA, April 1997
d) NATO Workshop, Jesenik, Czech Republic, August 1997
e) Minisymposium on Biosorption and Microbial Degradation V, Prague, Czech Republic, November 1997
f) Second International Symposium Biosorption and Bioremediation, Prague, Czech Republic, July 1998
g) Sixth International Mycological Congress, Jerusalem, Israel, August 1998
h) Workshop der VAAM-Fachgruppe Mykologie, Friedrich-Schiller-Universitat Jena, Germany, October 1998

i) Silver Jubilee of the Mycological Society of India & Asian Mycological Congress, International Symposium on Tropical Mycology, University of Madras, India, January 1999
j) Minisymposium on Biosorption and Microbia Degradation VI, Sec u Chriudimi, Czech Republic, March 1999
k) In Situ and On-Site Bioremediation, The Fifth International Symposium, San Diego, Colifornia, USA, April 1999

2 *Training*

i) Two scientists from the Czech laboratory (A Kubatova and C Novotny) spent a two months training stay in MIGAL in the fall 1996 to learn laboratory solid-phase cultivation techniques and carry out *in vitro* and *in vivo* biodegradation experiments
ii) In 1998 training of two coworkers of Czech laboratory in MIGAL (P Erbanova 8 weeks, C Novotny 2 weeks) was directed to the study of production of ligninolytic enzymes in the model fungus *Irpex lacteus* on lignocellulosic substrate and in soil It enabled to take over into Czech laboratory the methods routinely used in MIGAL laboratory for estimation of specific activities of individual enzymes of the ligninolytic complex
iii) In March 1999 C Novotny participated one week on Practical Training Course on Toxkits in Besse, France, that was partially supported from the grant The reason of the stay was getting the experience with the Toxkit method of biological toxicity tests

3 *Publications*

i) In reviewed journals

Kubatova A , Matucha M , Erbanova P , Novotny Č , Vlasakova V , Šašek V Investigation into PCB biodegradation using uniformly 14C-labelled dichlorobiphenyl Isotopes Environ Health Stud 34 325-334, 1998
Šašek V , Novotny Č , Vampola P Screening for efficient organopollutant fungal degraders by decolorization Czech Mycol 50 303-311, 1998
Novotny Č , Erbanova P , Šašek V , Kubatova A , Cajthaml T , Lang E , Krahl J , Zadrazil F Extracellular oxidative enzyme production and PAH removal in soil by exploratory mycelium of white rot fungi Biodegradation 00 1-10, 1999
Krčmař P , Kubatova A , Votruba J , Erbanova P , Novotny Č , Šašek V Degradation of polychlorinated biphenyls by ligninolytic enzymes of *Phanerochaete chrysosporium* produced in perforated plate bioreactor World J Microbiol Biotechnol (in press)
Matucha M , Bubner M , Kubatova A , Erbanova P , Uhlířova H , Novotny Č , Šašek V Methodical aspects of application of 14C-labelling in ecological research Acta Univ Carolinae 1999 (in press)
Kubatova A , Erbanova P , Volakova I , Nerud F , Šašek V PCB congener selective biodegradation by the white rot fungus *Pleurotus ostreatus* in contaminated soil Chemosphere (in preparation)
Č Novotny, P Erbanova, T Cajthaml, M Bhatt, N Rothschild, C G Dosoretz, and V Šašek Irpex lacteus, a white rot fungus applicable to water and soil bioremediation Appl Microbiol Biotechnol (in preparation)
N Rothschild, C Novotny, V Sasek and C G Dosoretz Ligninolytic enzymes of the fungus *Irpex lacteus* (*Polyporus tulipiferae*) Isolation and characterization of lignin peroxidase Appl Environ Microbiol (in preparation)

ii) Proceedings

Šašek V , Novotny C , Erbanova P , Bhatt M , Cajthaml T , Kubatova A , Dosoretz C , Rawal B , Molitoris H P Selection of ligninolytic fungi for biodegradation of organopollutants Proceedings of the In Situ and On-Site Bioremediation, The Fifth International Symposium, April 19-22, San Diego, California, USA,1999 (in press)
Kubatova A , Erbanova P Factors affecting the evaluation of long-term PCB biodegradation in soil by white rot fungi , pp 141-146 in Baveye Ph , Block J -C- Goncharuk V V (Eds) Bioavailability of organic xenobiotics in the environment - Practical consequences for the environment NATO ASI Series, Kluwer Publishers, 505 pp, 1999 (in press)

c) Lectures or posters on scientific meetings with published abstracts

Šašek V , Novotny Č , Erbanova P Selection of ligninolytic wood -rot fungal strains based on decolorization of poly R-478 and remazol brilliant blue R (RBBR) dyes Abstr p 73, 8th IUMS Congress, Jerusalem 1996
Erbanova P , Novotny Č , Kubatova A , Cajthaml T , Šašek V Comparison of the ability of various white rot fungi to degrade PCBs and PAHs in soil Abstract pp 49-51, Mini-Symposium on Biosorption and Microbial Degradation V, Prague, November 24-28, 1997
Kubatova A , Erbanova P , Matucha M , Šašek V Effect of PCB sorption and volatility on biodegradation experiments in soils Abstract pp 21-22, Mini-Symposium on Biosorption and Microbial Degradation V, Prague, November 24-28, 1997
Šašek V , Novotny Č , Erbanova P , Kubatova A , Cajthaml T Fate of PCBs and PAHs during their degradation by white rot fungi in soil Abstract pp 32-33, Mini-Symposium on Biosorption and Microbial Degradation V, Prague, November 24-28, 1997
Cajthaml T , Kubatova A , Erbanova P , Pacakova V Application of supercritical fluid extraction (SFE) in the study of biodegradation in soil Abstract P 4-2, 2nd International Symposium Biosorption and Bioremediation, Prague, Czech Republic, July 12-17, 1998
Novotny Č , Erbanova P Cajthaml T , Kubatova A , Bhatt M , Dosoretz C , Rawal B , Molitoris H P , Šašek V Irpex lacteus, a white rot fungus (WRT) with significant biodegradation potential Abstract L 3-12, 2nd International Symposium Biosorption and Bioremediation, Prague, Czech Republic, July 12-17, 1998
Šašek V , Novotny Č , Erbanova P , Cajthaml T , Kubatova A Removal of PAHs and PCBs from contaminated soil by white rot fungi Abstr p 87 in Abstracts of the 6th Internat Mycol Congr , Jerusalem, Israel, August 23-28, 1998
Šašek V , Novotny Č Soil bioremediation using white rot fungi strong and weak points Abstr p 23 in Workshop der VAAM-Fachgruppe Mykologie, Friedrich-Schiller - Universitat Jena, 8 -9 October 1998
Šašek V , Novotny C , Erbanova P , Bhatt M , Cajthaml T , Kubatova A , Dosoretz C , Rawal B , Molitoris H P Selection of ligninolytic fungi for biodegradation of organopollutants Abstr B6 in In Situ and On-Site Bioremediation, The Fifth International Symposium, April 19-22, San Diego, California, USA 1999
Novotny Č , Šašek V , Molitoris H P Biodegradace aromatickych uhlovodiku a syntetickych barviv houbami aktivně rostoucimi v tekutych mediich a v pudě Abstr p 18 in VI ročnik Minisympozia o biosorpcich a biodegradacich a III ročnik seminaře Biodegradace, Sbornik sympozia, Seč u Chrudimi 8 -11 března 1999
Novotny C , Rawal B , Bhatt M , Patel M , Sasek V , Molitoris M Fungal degradation of synthetic dyes Abstr pp 44-46 in Silver Jubilee of the Mycological Society of India & Asian

Mycological Congress, International Symposium on Tropical Mycology, University of Madras, India, 20-22 January, 1999

iv) Dissertations

Kubatova A Optimization and application of analytical methods in biodegradation studies of polychlorinated biphenyls (PCBs) /in Czech/ PhD-thesis, Faculty of Science, Charles University, Prague 1997
Cajthaml T Application of analytical methods in the study of PAH degradation /in Czech/ Ms-thesis, Faculty of Science, Charles University, Prague 1998

PROJECT PRODUCTIVITY

The project was from the beginning planned with the aim to get maximum from the study of physiology of white rot fungi and their possible application in soil remediation All the main parts were fulfilled as it was originally proposed The problem raised with the introduction of biological toxicity tests We lost the time trying to introduce, as the first test, the method involving luminescence bacteria Since the target compounds (PAHs and PCBs) are practically water insoluble, we did not succeeded to overcome this factor That is why in the last running experiment we are using another solid-phase microbial toxicity test based on a specific enzymatic activity of *Bacillus cereus* which reduces a pH indicator dye (resazurin) This method had not been known in the time when the project was prepared On the other hand we included the treatment of contaminated soil by composting that was not originally involved in the program

FUTURE WORK

The work started and developed during this project will continue The Czech laboratory will prolong the work on the basis of the grant of the Grant Agency of the Czech Republic "Comparison of the effectiveness of ligninolytic fungi-assisted microbial remediation of polluted soils with the method of composting" for the period 1998-2000 All analytical equipment purchased during the AID grant will be used in the forthcoming research work

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