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Screening of *Pleurotus ostreatus* isolates for their ligninolytic properties during cultivation on natural substrates

Ivana Eichlerová[1,*], Ladislav Homolka[1], František Nerud[1], František Zadrazil[2], Petr Baldrian[1] & Jiří Gabriel[1]

[1] *Institute of Microbiology AS CR, Vídeňská 1083, 142 20 Prague 4, Czech Republic;* [2] *Institute of Plant Nutrition and Soil Science, FAL, Bundesallee 50, D-38116 Braunschweig, Germany (* author for correspondence, e-mail: eichler@biomed.cas.cz)*

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Abstract

Thirteen basidiospore-derived isolates of *Pleurotus ostreatus* f6 strain differing in the level of ligninolytic enzyme production and other characteristics (mycelium extension rate, colony morphology) from the parental strain were cultivated on natural substrates. Under these conditions ligninolytic enzyme activity, loss of organic mass, polycyclic aromatic hydrocarbons (PAHs) degradation and colonization of sterile and nonsterile soil were studied. The activity of ligninolytic enzymes was substantially higher in straw than in liquid culture, although the differences between the isolates were less pronounced on this substrate. Some of the isolates showed a very good ability to decompose the lignocellulosic substrate (straw) and a relatively high loss of organic mass was found after 50 days of cultivation in these strains. The original strain f6 and isolates B13 and B26 successfully degraded all seven tested PAH compounds present in experimental soil samples, but the higher or lower ligninolytic enzyme production of isolates tested had no substantial effect on the extent of the degradation. In our screening, six basidiospore-derived isolates growing well in nonsterile soil were found, which could be suitable for the prospective biotechnological exploitation.

Abbreviations: ABTS – 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid, DMAB – 3-dimethylaminobenzoic acid, EDTA – ethylendiaminetetraacetate, MBTH – 3-methyl-2-benzothiazolinone hydrazone, MnP – manganese peroxidase, PAH – polycyclic aromatic hydrocarbon

Introduction

The process of lignin biodegradation represents a key role in the carbon cycle on Earth. In nature, lignin is metabolized by several groups of organisms, among which white-rot fungi belonging to basidiomycetes have been intensively studied in recent years. White-rot fungi destroy lignin more extensively and quickly than any other known group of organisms. In contrast to other fungi and bacteria they are capable of complete lignin degradation to carbon dioxide and water (Kirk et al. 1978). The high biodegradation capacity of white-rot basidiomycetes is assumed to result from the activities of numerous ligninolytic enzymes secreted by these fungi. Recent literature emphasizes the role of hydrogen peroxide and free radicals in lignin biodegradation (Tanaka et al. 1999; Kapich et al. 1999). Although the exact role of individual enzymes involved in the process of lignin decomposition is not yet fully understood, two families of ligninolytic enzymes are widely considered to play a key role in this process: phenol oxidases (laccase) and peroxidases (lignin peroxidase, manganese peroxidase). *Pleurotus ostreatus* belongs to the group of white-rot fungi producing laccase and manganese peroxidase

but not lignin peroxidase. Laccase catalyzes the one-electron oxidation of various aromatic compounds coupled to the four-electron reduction of molecular oxygen. With respect to these catalytic properties, the *P. ostreatus* manganese-dependent peroxidase in addition to oxidize Mn^{2+} as the best substrate, is able to oxidize phenolic (2,6-dimethoxyphenol) and non-phenolic substrates (veratryl alcohol) in the absence of Mn^{2+} (Sarkar et al. 1997). Some ligninolytic enzyme-producing organisms including *P. ostreatus* are able to degrade environmental pollutants, e.g., polycyclic aromatic hydrocarbons (Bumpus 1985, 1989; Haemmerli 1986).

White-rot fungi are often used to convert lignocellulosic material into feed (Zadrazil 1985, 1997) and for the decontamination of organopolluted soil (in der Wiesche et al. 1996; Lang et al. 1998). One of the important attributes of biotechnologically interesting strains is their ability to colonize natural substrates. Fast and complete colonization of agricultural plant residues such as straw is mostly the first precondition for their effective delignification by ligninolytic microorganisms. Several environmental factors influence the process of lignin decomposition. Among these, the effect of temperature seems to be most important for biotechnological applications.

Previous papers (Homolka et al. 1995; Eichlerová-Voláková & Homolka 1997) showed that apart from classical mutagenization, the preparation of protoplast-derived isolates or basidiospore-derived monokaryotic isolates is an efficient method of achieving a higher variation in the production of the enzymes involved in lignin degradation by *P. ostreatus*. Using a modified technique of fructification of *P. ostreatus* under laboratory conditions (Eichlerová-Voláková & Homolka 1997), we obtained several sets of basidiospore-derived isolates. Their detailed study indicated that this method could be very efficient for the preparation of isolates with changed ligninolytic characteristics. The variation of enzyme activities (especially laccase) found in isolates derived from basidiospores increased substantially compared with the control set and was even higher than that of isolates obtained after UV mutagenesis (Eichlerová-Voláková & Homolka 1997). In comparison with the classical mutagenesis, one of the main advantages of these alternative methods is the elimination or reduction of concomitant undesirable changes in the properties of the strains tested. Mutual crossing of compatible basidiospore-derived monokaryons gave rise to dikaryotic isolates with new interesting combinations of characteristics. All the isolates were morphologically and biochemically characterized and repeated tests confirmed a relative stability of the changes.

The high levels of ligninolytic enzymes in some of the monokaryotic isolates, and in the dikaryons prepared by the crossing of these isolates observed in liquid culture, is a promising prerequisite for their use in environmental biotechnology. In our present work, we studied the behavior of the strains in this respect. Therefore, the growth and activity of ligninolytic enzymes were studied not only on artificial media, but also by using straw as a natural substrate. In addition, the growth of the strains was also tested in sterile and nonsterile soil to estimate their potential for in situ degradation of xenobiotics like polycyclic aromatic hydrocarbons.

Materials and methods

Organisms

Pleurotus ostreatus Florida f6 (Block et al. 1959) obtained from the Department of Plant Pathology and Microbiology, Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University of Jerusalem, Israel, and its derivatives were maintained by serial transfers and kept on wort agar slants at 4 °C.

Soil samples

The soil collected from the upper 15-cm layer of an agricultural site near Braunschweig (Germany) was used in this study. The soil sample (sandy loam, C_{org} 0.8%, pH 5.3) was sieved (<2 mm), moistened to 45% of its maximum water-holding capacity and left undisturbed at 22 °C for 1 week before it was frozen at –25 °C. Seven days before application, the required amount of soil was removed from the freezer and incubated for 2 days at 4 °C and a further 5 days at 25 °C (in der Wiesche et al. 1996).

Estimation of ligninolytic enzyme activity in liquid culture

Static submerged cultivation was carried out in 100-mL Erlenmayer flasks with 10 mL of N-limited Kirk medium (Tien & Kirk 1988) with veratryl alcohol (final concentration 1 mM). The flasks were inoculated with two wort agar plugs (10-mm diameter) cut from the actively growing part of the colony on a Petri dish

and incubated for 10, 14 or 18 days at 27 °C. The enzyme activity was measured in the filtrates from four parallel flasks after removing the mycelia. The mycelia were used for dry mass determination. All measurements were repeated three times.

Estimation of ligninolytic enzymes activity during the growth on straw

Cultivation was carried out in 100-mL Erlenmeyer flasks containing 5 g of air-dried milled wheat straw (particle size <1 mm). The straw was moistened with 15 ml of deionized water (content 75%) and autoclaved (121 °C for 40 minutes). The straw was then inoculated with two 10-mm agar plugs cut from the actively growing part of the colony (strains f6, B13 and B26) and incubated at 22, 25 and 30 °C for 26 days. At the end of incubation, the straw was quantitatively transferred into a 60-ml centrifugation cuvette and supplemented with 30 ml of 160 mM acetate buffer (pH 5.0). The content of each cuvette was then mixed thoroughly and incubated for 3 hours in the ice bath. The samples were centrifuged at 15 500 g at 15 °C for 15 minutes and the supernatant subsequently at 5500 g at 15 °C for 15 minutes. The resulting supernatant was used to determine the enzyme activities. Four replicates were used for each strain and temperature.

Enzyme assays

The activities of laccase and manganese peroxidase (MnP) were determined spectrophotometrically by monitoring the absorbance increase at 425 nm (laccase) or 590 nm (MnP) in the medium filtrates. The laccase activity was determined according to Bourbonnais and Paice (1990) by monitoring the oxidation of ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)). The determination of the activity of MnP was based on the Ngo and Lenhoff (1980) method modified according to Daniel et al. (1994). MBTH (3-methyl-2-benzothiazolinone hydrazone, Sigma) and DMAB (3-dimethylaminobenzoic acid, Sigma) were oxidatively coupled to give a purple indamine dye product by the action of the enzyme in the presence of added H_2O_2 and Mn^{2+} ions. The values were corrected for present laccase activity. One unit of enzyme activity (U) was defined as the amount of enzyme catalyzing the production of 1 μmol of green or purple dye per ml/min.

Estimation of growth rate on Petri dishes

As a criterion for the growth of isolates on solid media mycelium, an extension rate was used. This was estimated by measuring the diameters of four individual colonies grown separately on the solid agar Kirk medium (pH 5.5) in Petri dishes inoculated with agar plugs (1-mm diameter) cut with an injection needle from the actively growing part of colony on another Petri dish. All measurements were repeated three times and done in quadruplicate.

Determination of growth rate in straw and soil

Glass tubes (inner diameter 30 mm) were filled with 25 g of air-dried milled wheat straw (particle size <1 mm), forming a 60-mm column, then supplemented with 2 ml of water and sterilized by autoclaving (45 minutes at 120 °C, 101 kPa). The tubes were then inoculated with a 10-mm agar plug cut from the actively growing part of the mycelial colony. After the establishment of fungal mycelia in the straw compartment, 25 g of soil was added to the straw surface to form a 60-mm layer. Nonsterile and sterile soil (twice autoclaved for 45 minutes at 120 °C, 101 kPa) was used in this experiment. All the cultivations proceeded in 22, 25 and 30 °C. During the cultivation, the fungal growth was assessed daily by measuring the visible penetration of mycelia into the straw or soil (Lang et al. 1997). After 14 days of growth the colonization ability of each strain was expressed as the percentage of the height of the colonized straw column in proportion to the total height of the straw column. Three replicates were used for each treatment.

Determination of the loss of organic mass

Erlenmeyer flasks (500 ml) containing 25 g of air-dried milled wheat straw (particle size <1 mm) were sterilized by autoclaving (45 minute at 120 °C, 101 kPa) and inoculated with two 10-mm agar plugs cut from the actively growing part of the mycelial colony. The flasks were then incubated for 30 or 50 days at 22 or 30 °C. At the end of the cultivation, the content of each flask was dried at 100 °C until the constant weight, weighed and the loss of organic mass was determined. The dry mass of sterile straw (22.74 ± 0.25 g) cultivated under the same conditions without fungus was used as a control. Three replicates were used for each strain and treatment.

Degradation of PAHs in soil

Cultivation was carried out in 100-mL Erlenmeyer flasks containing 5 g of air-dried milled wheat straw (particle size <1 mm). The straw was moistened with 15 ml of deionized water (content 75%), covered with a nylon mesh and autoclaved (121 °C for 40 minutes). The cultures were inoculated with two 10-mm agar plugs cut from the actively growing part of the mycelial colony of *P. ostreatus* f6 and incubated at 22, 25 and 30 °C until the mycelia had completely colonized the substrate (14 days). Then 12.25 g of moist soil (corresponding to 10.75 g of dry matter, water content 14.0%) was spread on the surface of each straw culture. To improve contact between the soil and straw compartments, 1 ml of deionized water was added dropwise on the surface of the soil. Before use, the soil for each flask was supplemented with PAH by mixing it with 0.75 g of dry soil supplemented with 150 μl of PAH solution in toluene containing pyrene, benzo[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene (all from Aldrich) and dibenzo[*a,h*]anthracene (Sigma). The final concentration of each compound in the respective flask (i.e., in 10.75 g of dry soil) was 10 μg g^{-1} dry soil. All the flasks were incubated for 15 weeks at 22, 25 and 30 °C. After incubations, the flasks were collected and dried at 60 °C until they reached constant weight. The soil layer was then separated from the straw and used for PAHs determination. Two controls were included in all experiments – one to obtain the initial PAH value (PAH-supplemented soil was spread on the fungal straw cultures as described above and then immediately recovered, dried and used for estimation), and the other – a true abiotic control (sterile straw-soil system without fungus), to which all the results were referred.

Determination of PAH concentration

The dried soil samples were homogenized with a mortar and pestle before extraction. Five grams of homogenized soil were extracted using a Soxhlet apparatus with acetone : *n*-hexane (1 : 4) (Merck) for 6 hours at 76 °C. The extracts were evaporated and resuspended in 20 ml of acetonitrile (Merck). The HPLC system used for PAHs estimation consisted of a liquid chromatograph HP 1090L (Hewlett-Packard) and fluorescence detector HP 1046A (Hewlett-Packard). Separations were performed at 25 °C isocratically with an analytical column of 150 ± 4.6 mm inner diameter (Hypersil PAH 5 μm). A mixture of acetonitrile and water (1000 : 1) was used as a mobile phase at a flow rate of 0.5 ml min^{-1}. Fluorescence detection was performed using excitation wavelength 270 nm and emission wavelength 405 nm. Each sample was analyzed twice. The concentration of PAHs was determined using calibration with a mixture containing all the PAHs involved in the experiment. The extraction efficiency of the samples was above 85% for all the individual PAH compounds.

Results and discussion

Ligninolytic enzyme activity and growth rate on artificial media

Original strain of *P. ostreatus* f6 (control) and 13 basidiospore-derived isolates with different ligninolytic enzyme activity during cultivation on low-nitrogen Kirk medium were chosen for our work. *Pleurotus ostreatus* f6 and its basidiospore-derived isolates produced laccase and manganese peroxidase. Lignin peroxidase was not detected (Hadar et al. 1993). In repeated experiments the maximum production of laccase occurred mostly on day 10 of cultivation and the maximum production of MnP on day 14. Table 1 shows the activity of laccase found on day 10 or MnP found on day 14. The isolates also showed remarkable changes in the growth rate (determined as a mycelium extension rate on complete GC 3% agar medium) in comparison with the control strain. Isolates with good ligninolytic enzyme production usually showed a rather lower growth rate than the control strain. However, three dikaryotic isolates obtained after the crossing of compatible monokaryons showed a higher growth rate than the control strains together with higher enzyme activity (Table 1).

Colonization and degradation of straw

Table 2 summarizes the results of straw colonization experiments. All the tested isolates were slower to colonize the straw than the control strain. Eleven out of 13 tested isolates exhibited the maximum growth rate at 30 °C. The same result was found in the control strain *P. ostreatus* f6. However, the growth rates of two isolates (B2 and B4xB18) were found to decrease significantly with increasing temperature. The results indicate a positive correlation between the growth rate tested on GC 3% agar medium and on the natural substrate (straw). A higher growth rate on the agar plates corresponded to a higher ability to colonize straw and

Table 1. Characterization of basidiospore-derived isolates under cultivation on synthetic media

Isolate number	Maximum activity of ligninolytic enzymes on low-nitrogen Kirk medium (% control)		Growth (% of control)[a]	Dry mass (% of control
	Laccase	MnP		
B2	1338	137	46	45
B13	2597	403	56	32
B20	1081	32	71	45
B26	34	36	51	56
B29	2865	65	47	27
B4xB18	789	175	116	69
B13xB5	5892	666	26	43
B13xB11	1768	197	36	55
B13xB12	824	168	117	74
B13xB18	2869	266	27	50
B13xB19	1048	62	64	44
B13xB20	983	43	59	65
B13xB21	1003	120	120	94
Control[b]	56.87 ± 11.61	118.67 ± 19.22	14.25 ± 1.5[c]	6.6 ± 0.4[d]

[a] Growth of isolates, measured as mycelium extension rate on complete GC 3% agar medium.
[b] Enzyme production of control strain *P. ostreatus* f6 (U mg^{-1} of dry mass) $\times 10^{-4}$.
[c] Mycelium extension rate of the control strain (mm day^{-1}).
[d] Dry mass of control strain *P. ostreatus* f6 (mg ml^{-1}).

Table 2. Ability of *P. ostreatus* f6 and its basidiospore-derived isolates to colonize sterile straw

Isolate No.	Growth rate (mm day^{-1})		
	22 °C	25 °C	30 °C
f6 original strain	3.26 ± 0.16	4.51 ± 0.28	4.86 ± 0.15
B2	1.07 ± 0.08	0.71 ± 0.15	0.55 ± 0.06
B13	0.92 ± 0.21	1.14 ± 0.16	2.07 ± 0.16
B20	1.50 ± 0.27	1.86 ± 0.12	2.00 ± 0.18
B26	1.43 ± 0.15	1.25 ± 0.06	1.64 ± 0.10
B29	0.71 ± 0.15	1.29 ± 0.16	1.57 ± 0.07
B4xB18	2.50 ± 0.25	1.79 ± 0.25	1.21 ± 0.10
B13xB5	0.50 ± 0.15	0.75 ± 0.19	0.81 ± 0.11
B13xB11	1.43 ± 0.06	1.45 ± 0.17	1.67 ± 0.11
B13xB12	1.79 ± 0.22	2.10 ± 0.28	2.79 ± 0.19
B13xB18	1.14 ± 0.06	1.06 ± 0.12	1.21 ± 0.08
B13xB19	0.57 ± 0.05	2.14 ± 0.26	2.50 ± 0.20
B13xB20	2.24 ± 0.26	2.50 ± 0.15	2.77 ± 0.15
B13xB21	1.29 ± 0.21	1.57 ± 0.25	1.78 ± 0.18

the isolates with the low growth rate on medium also grew slowly on straw.

The extent of decomposition of plant polymers was found to depend on the fermentation temperature (Zadrazil & Brunnert 1981). This is also true for the studied isolates cultivated at 22 and 30 °C (Table 3). The majority of isolates caused a higher loss of organic mass during cultivation at 30 °C and only three (B4xB18, B13xB11 and B13xB18) degraded the substrate more effectively at 22 °C. The degradation of straw was much more pronounced at the lower temperature, particularly in the isolate B4xB18 where faster straw colonization was found at 22 °C. In four dikaryotic isolates and in the control strain *P. ostreatus* f6, the degradation of straw reached more than 30% within 50 days. In the case of isolates B13xB19 (at 30 °C), B13xB12 (at 30 °C) and B4xB18 (at 22 °C), the loss of organic mass was even higher than in the control strain. Zadrazil (1985) similarly tested 235 different strains and also found the higher loss of organic mass mostly at 30 °C. In his studies no more than 23.3% of straw dry weight was degraded by any *P. ostreatus* strain within 60 days. A strong correlation was found between the growth rates of the strains in straw columns and the degradation of straw.

Production of ligninolytic enzymes in straw

The control strain *P. ostreatus* f6 and two isolates, B13 with higher and B26 with lower enzyme activity than the control strain (measured on Kirk medium), were tested for ligninolytic enzyme production during cultivation on sterile straw for 26 days. Experiments were also carried out at three temperatures (22, 25 and 30 °C).

The results summarized in Table 4 indicate that different temperatures did not have substantial influence on enzyme production in tested strains. Enzyme activities reached their maxima on day 12 or 20. Isolate B26 exhibited lower enzyme activities than the control strain and B13, but differences between isolate B13 and the control strain were not as marked during the cultivation on straw as during the cultivation on Kirk medium. Moreover, whereas in liquid medium MnP and laccase activities were higher in B13 than in f6, in straw the activity of laccase was similar and MnP activity was even higher in the control strain. The absolute values of ligninolytic enzyme activities of strains cultivated on straw were substantially higher than activities reached during cultivation on Kirk medium. The increase of ligninolytic enzyme production during cultivation on straw was more significant in the case of the control strain (laccase: 2–3 times higher; MnP 32–58 times higher at three different temperatures tested), and in enzyme low-producing isolate B26 (laccase: 1.5 – 3.6 times higher; MnP: 5.7–31.8 times higher at three different temperatures tested), than in the case of isolate B13 (laccase: no increase; MnP: 3.9–7.8 times higher at three different temperatures tested). Our data indicate that the changes in the ligninolytic enzyme characteristics of isolates tested probably have a regulation character and can be influenced by different physiological conditions. The results show that the natural lignocellulosic substrate (straw) influenced enzyme activity positively but not all the changes in ligninolytic properties detected during cultivation of isolates in liquid N-limited Kirk medium were preserved during the cultivation on straw. Enhancement of the ligninolytic enzyme activity during cultivation on natural substrate is not surprising. Ardon et al. (1996) demonstrated a significant increase of laccase activity in liquid cultures of *P. ostreatus* after the addition of cotton stalk extract. Also, Vares et al. (1995) found a higher production of ligninolytic enzymes during cultivation of *Phlebia radiata* on wheat straw. According to some authors, the isoenzymes produced by white-rot fungi during solid substrate degradation might be coded for by different genes than enzymes produced in liquid culture (Datta et al. 1991; Lobos et al. 1994; Vares et al. 1995).

PAH degradation and colonization of sterile and nonsterile soil

In this work we studied the ability of the control strain *P. ostreatus* f6, isolate B13 (a monokaryotic isolate with higher production of ligninolytic enzymes on Kirk medium than the control strain) and isolate B26 (a monokaryotic isolate with lower production of ligninolytic enzymes) to degrade PAHs with 3–5 aromatic rings in soil. The results in Table 5 show that the degradation of seven tested PAHs (pyrene, benzo[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene and dibenzo [*a,h*]anthracene) was quite efficient. After 15 weeks incubation, pyrene was degraded to the highest extent: 85% of the initial amount by the control strain, 86% by isolate B13 and 90% by isolate B26. The lowest degradation was found in dibenzo[*a,h*]anthracene: 35% of the initial amount by the control strain, 21% by isolate B13 and 31% by isolate B26. The results show that the higher or lower ligninolytic enzyme

Table 3. Loss of organic mass during cultivation of *P. ostreatus* f6 and its basidiospore-derived isolates on sterile straw

Isolate No.	Loss of organic mass (%)			
	After 30 days of cultivation		After 50 days of cultivation	
	22 °C	30 °C	22 °C	30 °C
f6-original strain	9.07 ± 1.09	21.28 ± 1.42	27.28 ± 1.43	30.43 ± 3.18
B2	2.53 ± 1.52	2.00 ± 0.80	10.06 ± 0.71	12.17 ± 1.98
B13	5.21 ± 1.37	14.43 ± 3.53	19.88 ± 1.51	28.85 ± 3.05
B20	8.59 ± 0.94	14.82 ± 0.92	21.88 ± 1.21	26.27 ± 0.33
B26	1.64 ± 0.81	8.65 ± 1.04	10.82 ± 0.32	15.54 ± 1.02
B29	3.85 ± 1.58	4.10 ± 0.19	6.70 ± 1.40	6.96 ± 3.41
B4xB18	11.17 ± 1.24	4.33 ± 1.03	32.06 ± 2.03	11.79 ± 2.27
B13xB5	4.04 ± 0.37	4.98 ± 1.41	7.90 ± 1.92	15.46 ± 2.00
B13xB11	4.23 ± 1.81	6.59 ± 0.15	20.12 ± 0.24	12.73 ± 0.87
B13xB12	11.86 ± 0.52	8.34 ± 0.36	24.52 ± 1.37	31.04 ± 1.95
B13xB18	6.06 ± 1.10	6.52 ± 1.30	9.99 ± 0.76	7.36 ± 0.85
B13xB19	3.45 ± 0.91	15.15 ± 0.42	24.26 ± 0.83	35.51 ± 1.12
B13xB20	6.93 ± 0.88	8.62 ± 1.54	16.93 ± 0.51	22.01 ± 2.60
B13xB21	11.07 ± 1.67	10.84 ± 1.93	27.20 ± 1.69	30.25 ± 2.20

production has no substantial effect on the extent of degradation. This implies that some other factors (e.g., desorption from soil surface, H_2O_2 production etc.) or other enzymes may play a major role in the PAH biodegradation process. The rate-limiting step in the biodegradation of PAHs by white-rot fungi also seems to be their growth rate and ability to colonize contaminated soil. Therefore, we focused our attention on these characteristics.

All strains showed a higher growth rate in sterile than in nonsterile soil (Table 6). Whereas the most efficient colonization of sterile soil was mainly found at the highest temperature tested (30 °C), many strains colonized nonsterile soil more rapidly at 22 °C than at 30 °C. This could be due to the slower growth of competitive organisms (bacteria or other filamentous fungi – e.g., *Trichoderma* etc.) which optimal growth temperature is about 30 °C and higher. Five strains failed to grow in nonsterile soil. This is interesting, since different strains of *Pleurotus* sp. were repeatedly found to be good competitors when growing in nonsterile soil (Lang et al. 1997; Martens & Zadrazil 1998). The growth rates of individual strains in sterile and nonsterile soil are obviously independent, showing that the ability to colonize soil as a physical substrate and the ability to compete with indigenous microflora are unrelated. Good colonization of nonsterile soil was found in the control strain *P. ostreatus* f6 and in four dikaryotic isolates (B13xB12, B13xB19, B13xB20, B13xB21) at all temperatures tested, but only at 22 °C in dikaryotic isolate B4xB18 and only at 30 °C in monokaryotic isolate B13. Interestingly, these isolates had also the highest straw biodegradation ability and three of these isolates (B4xB18, B13xB12 and B13xB21) exhibited a higher growth rate than the control strain on defined media under laboratory conditions and had good (but not the highest) production of ligninolytic enzymes. More detailed study of enzyme production by these isolates under natural conditions will be the aim of further work.

The results obtained during this work confirmed that enzyme production, and some other characteristics determined under laboratory conditions, correlate only weakly with the situation in natural substrates. Particularly the ability or inability of a fungal strain to use straw as a substrate for growth and to grow in soil and compete with indigenous microflora may be the rate-limiting steps in the design of biotechnological remediation processes. Therefore, the screening methods based on the behavior of strains tested under natural conditions seem to be an essential step in the selection of biotechnologically interesting isolates prior to their introduction to contaminated sites.

Table 4. Enzyme activity of *P. ostreatus* f6, isolate B13 and isolate B26 during cultivation in low nitrogen Kirk medium and on sterile straw

Isolate/substrate		Maximum activity of ligninolytic enzymes (U ml^{-1}) × 10^{-2}	
		Laccase	MnP
f6 original strain	Kirk medium	3.25 ± 0.56	0.87 ± 0.05
	straw (22 °C)	9.63 ± 1.55	28.12 ± 4.85
	straw (25 °C)	6.98 ± 1.04	34.57 ± 6.01
	straw (30 °C)	6.35 ± 0.49	50.75 ± 1.31
B13	Kirk medium	68.93 ± 5.25	3.63 ± 0.85
	straw (22 °C)	9.90 ± 1.81	17.70 ± 2.45
	straw (25 °C)	10.40 ± 2.02	14.13 ± 3.05
	straw (30 °C)	6.27 ± 1.80	28.30 ± 1.91
B26	Kirk medium	1.41 ± 0.66	0.30 ± 0.25
	straw (22 °C)	5.01 ± 1.20	9.55 ± 2.08
	straw (25 °C)	2.06 ± 0.20	6.56 ± 4.19
	straw (30 °C)	2.99 ± 1.08	1.70 ± 0.47

Table 5. Degradation of PAHs in soil by original strain *P. ostreatus* f6 and isolates B13 and B26 obtained from basidiospores after 15 weeks incubation

Isolate	Degradation of PAHs (% initial amount)						
	Pyr	BaA	Chry	BbF	BkF	BaP	DaA
f6 original strain	85 ± 7	64 ± 4	42 ± 8	49 ± 8	48 ± 6	79 ± 3	35 ± 6
B13	86 ± 5	52 ± 3	29 ± 3	35 ± 2	31 ± 2	75 ± 2	21 ± 2
B26	90 ± 8	62 ± 1	38 ± 2	42 ± 5	41 ± 1	78 ± 4	31 ± 1

Compounds: Pyr = pyrene, BaA = benzo[*a*]anthracene, Chry = chrysene, BbF = benzo[*b*]fluoranthene, BkF = benzo[*k*]fluoranthene, BaP = benzo[*a*]pyrene, DaA = dibenzo[*a,h*]anthracene. Initial amount of each PAH: 10 μg g^{-1}.

Table 6. Ability of *P. ostreatus* f6 and its basidiospore-derived isolates to colonize sterile and nonsterile soil

Isolate No.	Growth rate (mm day^{-1})					
	22 °C		25 °C		30 °C	
	Sterile soil	Nonsterile soil	Sterile soil	Nonsterile soil	Sterile soil	Nonsterile soil
f6-original strain	4.11 ± 0.33	2.77 ± 0.16	4.22 ± 0.36	2.80 ± 0.28	4.71 ± 0.23	3.27 ± 0.08
B2	1.60 ± 0.35	0	0.84 ± 0.18	0	1.42 ± 0.21	0
B13	1.00 ± 0.23	0	1.58 ± 0.26	0	3.86 ± 0.30	1.42 ± 0.12
B20	1.94 ± 0.09	0	3.07 ± 0.24	0	2.72 ± 0.26	0
B26	3.18 ± 0.06	0	3.22 ± 0.29	0	3.48 ± 0.31	0.06 ± 0.02
B29	0.19 ± 0.17	0	1.06 ± 0.25	0	0.62 ± 0.09	0
B4xB18	4.46 ± 0.20	2.00 ± 0.21	4.08 ± 0.30	0	3.20 ± 0.26	0
B13xB5	0	0	1.28 ± 0.28	0	2.17 ± 0.20	0
B13xB11	2.26 ± 0.31	0.02 ± 0.01	3.09 ± 0.32	0.15 ± 0.01	2.71 ± 0.18	0.03 ± 0.01
B13xB12	1.36 ± 0.30	1.12 ± 0.22	1.45 ± 0.11	0.54 ± 0.11	3.64 ± 0.24	0.59 ± 0.24
B13xB18	0	0	0.14 ± 0.06	0	2.77 ± 0.23	0
B13xB19	1.97 ± 0.31	2.32 ± 0.20	1.92 ± 0.30	0.82 ± 0.08	3.55 ± 0.27	0.63 ± 0.23
B13xB20	2.12 ± 0.35	0.65 ± 0.20	1.73 ± 0.29	0.67 ± 0.22	4.65 ± 0.16	0.30 ± 0.12
B13xB21	2.30 ± 0.36	1.25 ± 0.29	2.18 ± 0.14	0.73 ± 0.12	4.70 ± 0.18	0.33 ± 0.10

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References

Ardon O, Kerem Z & Hadar Y (1996) Enhancement of laccase activity in liquid cultures of the ligninolytic fungus *Pleurotus ostreatus* by cotton stalk extract. J. Biotechnol. 51: 201–207

Block SS, Tsao G & Han L (1959) Experiments in the cultivation of *Pleurotus ostreatus*. Mushroom Sci. 4: 309–325

Bourbonnais R & Paice MG (1990) Oxidation of non-phenolic substrates. An expanded role for laccase in lignin biodegradation. FEBS Lett. 267: 99–102

Bumpus JA (1989) Biodegradation of polycyclic aromatic hydrocarbons by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 55: 154–158

Bumpus JA, Tien M, Wright D & Aust SD (1985) Oxidation of persistent environmental pollutants by a white-rot fungus. Science 228: 1434–1436

Daniel G, Volc J & Kubatova E (1994) Pyranose oxidase, a major source of H_2O_2 during wood degradation by *Phanerochaete chrysosporium, Trametes versicolor*, and *Oudemansiella mucida*. Appl. Environ. Microbiol. 60: 2524–2532

Datta A, Bettermann A & Kirk TK (1991) Identification of a specific manganese peroxidase among ligninolytic enzymes secreted by *Phanerochaete chrysosporium* during wood decay. Appl. Environ. Microbiol. 57: 1453–1460

Eichlerová-Voláková I & Homolka L (1997) Variability of ligninolytic enzyme activities in basidiospore isolates of the fungus *Pleurotus ostreatus* in comparison with that of protoplast-derived isolates. Folia Microbiol. 42: 583–588

Hadar Y, Kerem Z & Gorodecki B (1993) Biodegradation of lignocellulosic agricultural wastes by *Pleurotus ostreatus*. J. Biotechnol. 30: 133–139

Haemmerli SD, Leisola MSA, Sanglard D & Fiechter A (1986) Oxidation of benzo[*a*]pyrene by extracellular ligninases of *Phanerochaete chrysosporium*. J. Biol. Chem. 261: 6900–6902

Homolka L, Voláková I & Nerud F (1995) Variability of enzymatic activities in ligninolytic fungi *Pleurotus ostreatus* and *Lentinus tigrinus* after protoplasting and UV-mutagenization. Biotechnol. Techn. 9: 157–162

Kapich AN, Jensen KA & Hammel KE (1999) Peroxyl radicals are potential agents of lignin biodegradation. FEBS Lett. 461: 115–119

Kirk TK, Schultz E, Connors WJ, Lorenz LF & Zeikus JG (1978) Influence of culture parameters on lignin metabolism by *Phanerochaete chrysosporium*. Appl. Microbiol. 117: 277–285

Lang E, Eller G & Zadrazil F (1997) Lignocellulose decomposition and production of ligninolytic enzymes during interaction of white-rot fungi with soil microorganisms. Microb. Ecol. 34: 1–10

Lang E, Nerud F & Zadrazil F (1998) Production of ligninolytic enzymes by *Pleurotus* sp. and *Dichomitus squalens* in soil and lignocellulose substrate as influenced by soil microorganisms. FEMS Microbiol. Lett. 167: 239–244

Lobos S, Larrain J, Salas L, Cullen D & Vicuña (1994) Isoenzymes of manganese peroxidase and laccase produced by the lignin-degrading basidiomycete *Ceriporiopsis subvermispora*. Microbiology 140: 2691–2698

Martens R & Zadrazil F (1998) Screening of white-rot fungi for their ability to mineralize polycyclic aromatic hydrocarbons in soil. Folia Microbiol. 43: 97–103

Ngo TT & Lenhoff HM (1980) A sensitive and versatile chromogenic assay for peroxidase and peroxidase-coupled reactions. Anal. Biochem. 105: 389–397

Orth AB, Royse DJ & Tien M (1993) Ubiquity of lignin-degrading peroxidases among various wood-degrading fungi. Appl. Environ. Microbiol. 59: 4017–4023

Sarkar S, Martínez AT & Martínez MJ (1997) Biochemical and molecular characterization of a manganese peroxidase isoenzyme from *Pleurotus ostreatus*. Biochim. Biophys. Acta 1339: 23–30

Tanaka H, Itakura S & Enoki A (1999) Hydroxyl radical generation by an extracellular low-molecular-weight substance and phenol oxidase activity during wood degradation by the white-rot basidiomycete Trametes versicolor. J. Biotechnol. 75, 57–70

Tien M & Kirk TK (1988) Lignin peroxidase of *Phanerochaete chrysosporium*. Methods Enzymol. 161B: 238–248

Vares T, Kalsi M & Hataka A (1995) Lignin peroxidases, manganese peroxidases and other ligninolytic enzymes produced by *Phlebia radiata* during solid-state fermentation on wheat straw. Appl. Environ. Microbiol. 61: 3515–3520

in der Wiesche C, Martens R & Zadrazil F (1996) Two-step degradation of pyrene by white-rot fungi and soil microorganisms. Appl. Microbiol. Biotechnol. 46: 653–659

Zadrazil F (1985) Screening of fungi for lignin decomposition and conversion of straw into feed. Angew. Botanik 59: 433–452

Zadrazil F (1997) Changes in in vitro digestibility of wheat straw during fungal growth and harvest of oyster mushrooms (*Pleurotus* spp.) on laboratory and industrial scale. J. Appl. Anim. Res. 11: 37–48

Zadrazil F & Brunnert H (1981) Investigation of physical parameters important for the solid state fermentation of straw by white-rot fungi. Eur. J. Appl. Microbiol. Biotechnol. 11: 183–188