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Degradation of PAHs by Ligninolytic Enzymes of *Irpex lacteus*

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ABSTRACT. The ligninolytic fungus *Irpex lacteus* was shown as an efficient degrader of oligocyclic aromatic hydrocarbons (PAHs; 'polycyclic aromatic hydrocarbons') possessing 3–6 aromatic rings in complex liquid media. The strain produced mainly Mn-dependent peroxidase in media without pollutants. Activity of ligninolytic enzymes was higher in a N-limited medium. However, after contamination with PAHs (especially pyrene) the values increased and significant activity of Mn-independent peroxidase appeared in the complex medium. Other factors (such as the increase in nitrogen concentration or the presence of solvent(s) for dissolution of PAHs) had no effect. Cytochrome P-450 was detected in the microsomal fraction of biomass grown in the complex medium. The rate of PAH degradation was also affected by the presence of various combinations of PAHs. However, independently of the enzyme activities, anthracene was shown to have a positive influence on degradation of pyrene and fluoranthene.

Abbreviations

ANT	anthracene	CHR	chrysene
BaA	benz[*a*]anthracene	FLT	fluoranthene
BaP	benzo[*a*]pyrene	PHE	phenanthrene
BghiP	benzo[*g*,*h*,*i*]perylene	PYR	pyrene
LAC	phenol oxidase (laccase)	MIP	Mn-independent peroxidase
LiP	lignin peroxidase	MnP	Mn-dependent peroxidase
LNMM	low-nitrogen mineral medium	PAHs	oligocyclic aromatic hydrocarbons ('polycyclic aromatic hydrocarbons')
MEG	malt extract–glucose medium		

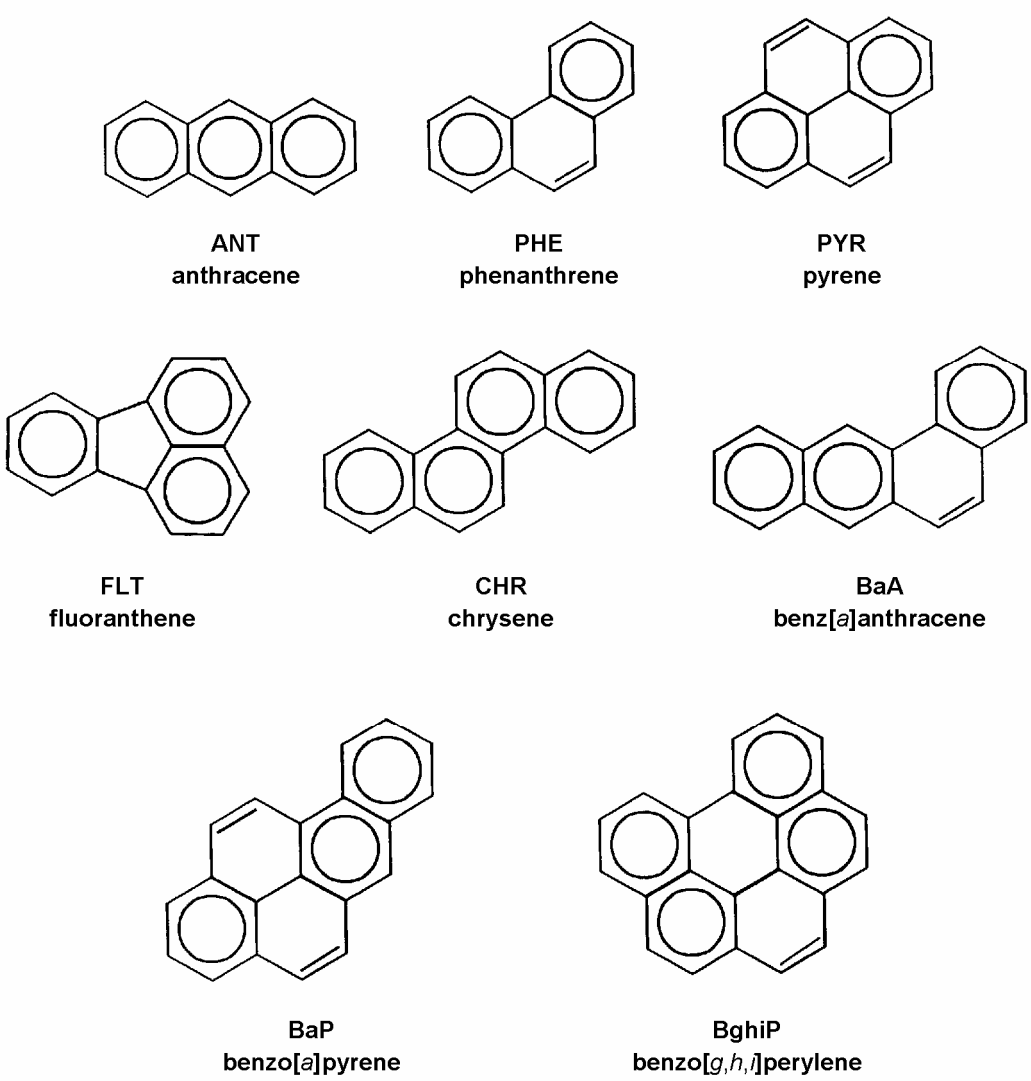
PAHs and some of their derivatives are a class of serious environmental pollutants, which often have a high mutagenic and carcinogenic potential. One way of removing PAHs from the environment is bioremediation using various types of organisms (Cerniglia 1992; Cajthaml *et al.* 2001) including ligninolytic fungi (Bhatt *et al.* 2002; Šašek 2003). These fungi produce extracellular enzymes with low substrate specificity, making them suitable for degradation of various compounds. The ligninolytic system consists of three major enzymes: LiP (EC 1.11.1.14), MnP (EC 1.11.1.13) and LAC (EC 1.10.3.2). Purified ligninolytic enzymes were *in vitro* able to extensively degrade PAHs (Hofrichter *et al.* 1998; Baborová *et al.* 2006). Although the degradation process of PAHs by ligninolytic fungi has been widely studied, involvement of the enzymes in the *in vivo* degradation remains unclear. Several papers (Sack and Günther 1993; Yadav and Reddy 1992; Novotný *et al.* 1997; Schutzendubel *et al.* 1999) put in doubt a direct correlation between the activity of ligninolytic enzymes and degradation of xenobiotic aromates. Therefore Field *et al.* (1993) and Šašek (2003) suggested that other "unidentified" enzymes must be involved in the oxidation of pollutants.

In ligninolytic fungi, similarly to other microorganisms (Stündl *et al.* 1998; George and VanEtten 2001), P-450 was also found and its participation in the degradation of PAHs was proved (Bezalel *et al.* 1996; Cerniglia *et al.* 1992). 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 for soil bioremediation (Lang *et al.* 1996; Bhatt *et al.* 2002; Novotný *et al.* 2004; Šnajdr and Baldrian 2006, 2007).

The aim of this paper was to investigate PAH biodegradation by the ligninolytic fungus *Irpex lacteus* (*1*) by stimulation of ligninolytic enzyme activities by PAHs, (*2*) by affecting the production of different enzyme systems with various culture media, and (*3*) by changing the degradation rate of individual pollutants by using their mixtures. These factors are considered to play an important role in elucidation of the degradation process.

MATERIALS AND METHODS

Chemicals. Anthracene, phenanthrene, pyrene, fluoranthene, chrysene, benz[*a*]anthracene, benzo[*a*]pyrene and benzo[*g*,*h*,*i*]perylene (*Fluka*, Germany) were used (Table I); they were applied without further purification to prepare stock solution in $HCONMe_2$. All solvents were purchased from *Merck* (Germany) and were of analytical grade, trace analysis quality or gradient grade, other chemicals were from *Sigma* (France).



Culture conditions. Static 20-mL cultures of *Irpex lacteus* (FR.:FR.) FR., strain 617/93 were incubated in 250-mL Erlenmeyer flasks in pentuplicates at 28 °C. The nutrient liquid media were used: LNMM containing 2.4 mol/L diammonium tartrate (Vyas *et al.* 1994), complex MEG containing (per L) 5 g malt extract broth (*Oxoid*, UK) and 10 g glucose (Novotný *et al*. 2000). The liquid cultures were inoculated with a suspension of homogenized, 1-week grown mycelium (5 %, *V*/*V*). In the degradation experiments the media were supplemented with the corresponding PAH at the time of inoculation. The cultures were spiked with a solution of the PAH in $HCONMe_2$ (150 μL). The final amount of each PAH was 0.5 mg per flask.

During incubation the samples were harvested up to 50 d. Degradation was performed in the simultaneous presence of PAHs included in the mixtures and also with the individual compounds or their combinations (to clarify their induction and/or inhibition effects). The abiotic controls were performed using 1-week grown mycelium killed by autoclaving.

Enzyme determination. LiP was assayed with veratryl alcohol as the substrate; MnP was determined with 3-dimethylaminobenzoic acid; 3-methyl-2-benzothiazoline hydrazone as the chromogen (Novotný *et al.* 1997). LAC was estimated with 2,2´-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) as the substrate (Niku-Paavola *et al.* 1988). MIP was calculated from the peroxidase activity of MnP assay detected in the absence

Table I. Physico-chemical properties of PAHs[a]

Hydrocarbon	Acronym	Formula	Molar mass g/mol	Melting point °C	Vapor pressure nPa	Water solubility µg/L	$\log k_{ow}$ [b]	Ionization potential aJ	CAS no.[c]
Phenanthrene	PHE	$C_{14}H_{10}$	178	101	18 000	1290	4.46	1.29	85-01-8
Anthracene	ANT	$C_{14}H_{10}$	178	218	200	73	4.45	1.19	120-12-7
Fluoranthene	FLT	$C_{16}H_{10}$	202	110	254 000	260	5.33	1.26	129-00-0
Pyrene	PYR	$C_{16}H_{10}$	202	150	886	135	5.32	1.21	206-44-0
Chrysene	CHR	$C_{18}H_{12}$	228	159	0.57	14	5.61	1.21	218-01-9
Benz[*a*]anthracene	BaA	$C_{18}H_{12}$	228	256	7.3	0.6	5.83	1.25	56-55-3
Benzo[*a*]pyrene	BaP	$C_{20}H_{12}$	252	179	15	3.8	6.04	1.15	50-32-8
Benzo[*g,h,i*]perylene	BghiP	$C_{22}H_{12}$	276	278	20	0.26	7.23	1.17	191-24-2

[a]Bogan and Lamar 1995; Cai *et al.* 2007; Weissenfels *et al.* 1992. [b]Octanol–water partition coefficient (concentration in the octanol-to-aqueous phase). [c]*Central Authentication Service.*

of Mn^{2+} ions (Novotný *et al.* 1997). One unit of enzyme produced 1 mmol of the reaction product per min under the assay condition at room temperature.

Microsomal fractions were isolated from 7-d-old cultures (Mougin *et al*. 1997). Briefly, the cultures kept for 1 h at 4 °C were filtered, the biomass was washed with cold potassium phosphate buffer (0.1 mol/L, 50 mL, pH 7.2), and disrupted in a Virtis 45 blender at 375 Hz in phosphate buffer (30 mL) supplemented with glycerol (200 g/L) and bovine serum albumin (1.5 g/L). 3 × 10- and 2 × 15-s bursts were separated by 20-s cooling periods in 1 min. The crude homogenate was filtered and centrifuged (1000 ***g***, 15 min). The supernatant was centrifuged (10 000 ***g***, 15 min, and 100 000 ***g***, 90 min), the supernatant being referred to as the *cytosolic fraction*. The pellets were then suspended in buffer (10 mL) and centrifuged again (100 000 ***g***, 90 min). The washed pellets, resuspended in phosphate buffer (0.1 mol/L, 3 mL) containing glycerol (300 g/L), EDTA (0.1 mmol/L) and reduced glutathione (0.1 mmol/L), were referred to as the *microsomal fraction*. Both fractions were stored for up to 2 weeks at –80 °C until used. Protein content was estimated using the *BioRad* kit for protein assay. Cytochrome P-450 was determined in both cytosolic and microsomal fractions using CO-binding spectra (Omura and Sato 1964).

Sample preparation and chemical analysis. The whole content (mycelium with the liquid) of each sample was homogenized with Ultraturrax-T25 (*Janke & Kunkel IKA-Labortechnik*, Germany), acidified to pH ≈2 and then extracted with five 10-mL portions of ethyl acetate. The extracts were dried with disodium sulfate and concentrated with a rotary evaporator. To enable HPLC analysis, an aliquot of the extract was mixed with acetonitrile 1 : 10 (*V*/*V*) and the mixture was used for injection. The degradation of PAHs was measured using a HPLC system (*Waters* 2695 Separations Module) equipped with a diode-array detector (*Waters* 2996). An isocratic program was applied with acetonitrile–water 9 : 1 (*V*/*V*) and PAHs were determined at 254 nm; they were separated on a LichroCart-PAH column filled with LichroSphere (250 × 5 mm, particle ∅ 5 µm; *Merck*, Germany).

RESULTS AND DISCUSSION

Degradation of individual PAHs with respect to culture conditions. The amounts of PAH (namely ANT, PHE, PYR, FLT) in the cultures decreased (Table II). Because analysis was performed on the whole cultures, it was assumed that biodegradation occurred. However, PYR and FLT appeared to be degraded only in MEG medium, whereas ANT and PHE disappeared in LNMM medium. Due to ionozation potential value PHE and FLT should not be degraded by ligninolytic system (Bogan and Lamar 1995) and the biodegradation could be attributed to cytochrome P-450. However, in the presence of intermediates (*e.g.*, glutathione) PHE and FLT can also be decomposed with purified MnP (Baborová *et al.* 2006).

To elucidate the possible effect of nitrogen amount in the medium, the level of degradation was determined in modified media. LNMM was enriched with a 10-fold concentration of diammonium tartrate and MEG was prepared with malt extract con-

taining mycological peptone (*Oxoid*, UK). Under the modified conditions we did not observe any change of the extent of degradation (*not shown*). This finding confirms that (in *I. lacteus* cultures) the ligninolytic conditions elicited by low nitrogen concentration have limited importance for PAH biodegradation (Kotterman *et al*. 1994).

Table II. Degradation of PAHs (recovery, %) by *I. lacteus* in MEG and LNMM media[a]

Days	Medium[b]	ANT	PHE	PYR	FLT
50	control	76.2 ± 3.6	93.5 ± 2.5	84.0 ± 3.1	80.8 ± 0.9
6	MEG	62.4 ± 4.7	80.6 ± 5.0	84.4 ± 6.8	86.3 ± 4.9
20		13.4 ± 2.3	40.1 ± 2.7	42.2 ± 4.5	60.0 ± 2.4
50		0.8 ± 0.7	7.7 ± 2.6	4.7 ± 3.8	46.0 ± 2.4
6	LNMM	73.0 ± 3.1	99.4 ± 4.5	97.9 ± 2.9	98.1 ± 3.7
20		45.5 ± 4.0	82.2 ± 3.0	93.8 ± 1.6	93.5 ± 3.2
50		22.3 ± 8.6	9.7 ± 0.8	83.0 ± 2.7	79.0 ± 2.0

[a]ANT – anthracene, PHE – phenanthrene, PYR – pyrene, FLT – fluoranthene.
[b]MEG – malt extract–glucose medium, LNMM – low-nitrogen mineral medium.

Enzyme production with respect to culture conditions. Activity of ligninolytic enzymes reached maximal values after 15–20 d (Table II). In contrast to results of the degradation, the enzyme activity values were found to be higher in LNMM without PAH supplementation. The addition of the ANT, FLT, PHE, and PYR mixture resulted in increased ligninolytic enzyme activity and (in the case of MEG medium) a significantly higher activity of MIP appeared; it indicates a possible role of MIP in the degradation of PYR and FLT, especially when the concentration of Mn (necessary for proper MnP function) was 25× higher in LNMM compared to MEG medium (*data not shown*).

For the influence of individual compounds on the enzyme activity in MEG *see* Table III. Particularly in the case of PYR, stimulation of MnP and MIP activities was observed. The solvent used ($HCONMe_2$) showed no effect on the enzyme activities (*not shown*). In some papers (Yang *et al*. 2003; De Souza *et al*. 2004; Kollmann *et al*. 2005; Canas *et al.* 2007), one-ring compounds were demonstrated to act as inducers of ligninolytic system. Such compounds were also showed to be products of PAH degradation (Cajthaml *et al*. 2002, 2006). The selection of fungal strains for bioremediation applications should probably not be based only on the enzyme activities or ability to degrade some screening model substrates, *e.g.*, poly R-478 (Šašek 2003).

Table III. Maximal activity (U/L) of ligninolytic enzymes of *I. lacteus* in liquid nutrient media and in the presence of PAHs

Medium[a]	PAH[b]	MnP	MIP	LiP	Lac
LNMM	none	273	10	20	5
	mixture	459	91	42	12
MEG	none	120	9	3	6
	mixture	260	397	44	11
	ANT	66	29	21	12
	PHE	120	26	7	5
	PYR	239	152	46	8
	FLT	123	34	2	6

[a]LNMM – low-nitrogen mineral medium, MEG – malt extract–glucose medium.
[b]ANT – anthracene, PHE – phenanthrene, PYR – pyrene, FLT – fluoranthene.

The presence of CHR, BaA, BaP, or BghiP did not affect the ligninolytic enzyme activity (*not shown*); it can be explained, *e.g.*, by their lower solubility that influences significantly availability of the compounds for organisms (Semple *et al*. 2003).

Cytochrome P-450 (taking part in degradation of some PAHs; Bezalel *et al*. 1996), was not detected in cultures of *I. lacteus* grown in LNMM, however, 780 pmol/mg protein was estimated in the microsomal

fraction of biomass grown in MEG medium. For the CO-binding spectrum (that proves the presence of active P-450) *see* Fig. 1. Much lower activity was detected in the cytosolic fraction (4.8 pmol/mg protein). We detected (Cajthaml *et al*. 2002) at *I. lacteus* several novel intermediates of PAHs degradation, probably connected with the participation of P-450 in their biodegradation. Nevertheless, using PAHs as substrates, no P-450 activity was detected in microsomal or cytosolic fractions regardless of the culture conditions.

Degradation of PAH mixtures. *I. lacteus* degraded efficiently BaA, BaP and BghiP in MEG medium (Table IV), CHR being not degraded in any case. A possible explanation may be its high ionization potential (*see* Table I) that can negatively affect one-electron oxidation performed by extracellular ligninolytic system of the fungus (Bogan and Lamar 1995). Particularly ANT had a positive effect on the degradation rate of PYR and FLT, however, independently of the activity of ligninolytic enzymes. On the other hand, CHR, despite its low water solubility, negatively affected the degradation rate of other PAHs.

Table IV. Recovery (%) of different combinations of PAHs degraded in MEG medium[a]

PAHs	ANT	PHE	PYR	FLT	CHR	BaA	BaP	BghiP
ANT+PYR+FLT	6.1 ± 5.2	–	2.8 ± 1.7	20.6 ± 2.2	–	–	–	–
ANT+PHE+PYR+FLT	0.8 ± 0.7	7.7 ± 2.6	4.7 ± 3.8	35.0 ± 2.4	–	–	–	–
PHE+PYR+FLT	–	12.0 ± 2.5	13.0 ± 4.6	42.0 ± 3.4	–	–	–	–
PYR+FLT	–	–	12.5 ± 2.1	46.0 ± 2.5	–	–	–	–
CHR+ BaA + BaP + BghiP	–	–	–	–	91.3 ± 5.4	57.7 ± 5.0	33.0 ± 6.5	76.1 ± 4.8
CHR+ BaA + BaP	–	–	–	–	94.0 ± 4.3	55.0 ± 5.9	30.0 ± 7.0	–
BaA + BaP	–	–	–	–	–	2.9 ± 0.6	2.8 ± 0.7	0
BaA + BaP + BghiP	–	–	–	–	–	2.6 ± 0.9	5.5 ± 0.8	62.6 ± 3.2
BaA+ BghiP	–	–	–	–	–	19.2 ± 0.5	–	65.6 ± 2.8
BaP+ BghiP	–	–	–	–	–	0	5.8 ± 0.5	57.5 ± 5.8
Individual	–	–	–	–	93.0 ± 3.2	0	0	24.4 ± 2.0

[a](–) – not tested.

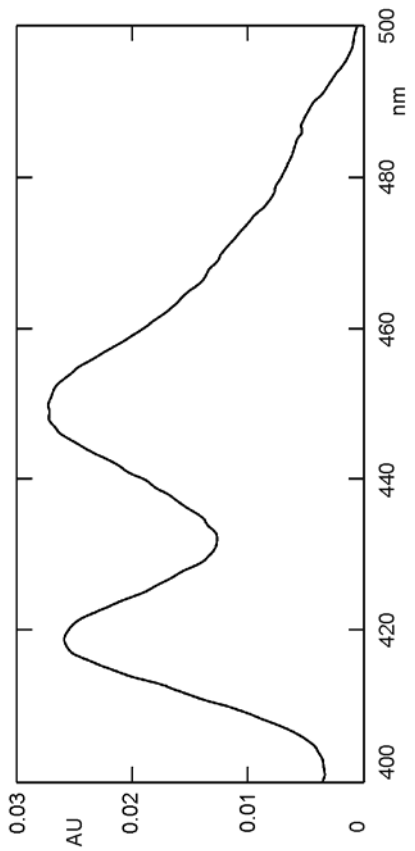


Fig. 1. Carbon monooxide-binding spectrum (nm) of cytochrome P-420 and P-450 of *I. lacteus* in MEG medium; AU – absorbance units.

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