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Screening for efficient organopollutant fungal degraders by decolorization

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A set of cultures of wood-degrading Basidiomycetes was screened for the ability to decolorize model synthetic dyes with the aim of selecting strains with the highest activities of ligninolytic enzymes. Four decolorization patterns were observed; some species possessed no decolorizing ability, some decolorized on all the media, some decolorized only when fully grown, and only a part of them followed a typical behaviour described in *Phanerochaete chrysosporium* Burds., *i.e.* decolorized only on nutrient limited media. The strains with the highest decolorizing capabilities will be further studied with respect to biodegradation of aromatic organopollutants.

Key words: decolorization, synthetic dyes, ligninolytic enzymes, white rot fungi, biodegradation.

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Soubor kultur dřevokazných bazidiomycetů byl testován z hlediska jejich schopnosti odbarvovat modelová syntetická barviva s cílem vybrat kmeny s nejvyšší aktivitou ligninolytických enzymů. Byly pozorovány čtyři typy odbarvování; některé druhy neodbarvovaly vůbec, některé bez ohledu na použité medium, některé až po úplné kolonizaci agaru na misce, a pouze část druhů se projevovala způsobem, který je popsán u *Phanerochaete chrysosporium* Burds., tj. pouze na mediu s limitovaným obsahem živin. Kmeny s nejvyšší dekolorizační aktivitou budou dále využity při studiu biodegradace aromatických organopolutantů.

Polymeric dyes like Poly R-478, Poly B-411 or Poly Y-606, which are not taken up by cells, can serve as suitable substrates for the detection of some enzymatic components of the fungal ligninolytic system. Degradation of those dyes correlates with the start of the lignin metabolism in white rot fungi and probably reflects a combined effect of peroxidases and H_2O_2-producing oxidases (Glenn and Gold 1983, 1985, Kuwahara et al. 1984; Paszczynski et al. 1991). Therefore, the dye-decolorizing test started to be used as a possible, easily usable and inexpensive alternative to radiolabelled lignin as a substrate in lignin biodegradation studies.

Recent investigations have shown that the enzymes of the ligninolytic complex are not only capable of efficeint degradation of lignin but also (probably) take part in the biodegradation of various recalcitrant xenobiotics, whose removal from contaminated soil and water is very difficult (Hammel 1989, Field et al. 1993).

Some studies demonstrated a good correlation between biodegradation of aromatic pollutants and decolorization of polymeric dyes (Field et al. 1992, 1993) and, therefore, this quick screening method can be applied for the search of prospective fungal degraders that can be used in soil remediation processes.

In this study, 39 new strains of wood-degrading fungi isolated from fruit bodies and decayed wood collected in forests in central Europe were tested on their efficiency to decolorize Poly R-478 and Remazol Brilliant Blue R dyes under various nutrient conditions in a solid agar medium.

MATERIAL AND METHODS

List of fungal strains tested

The cultures used in the study were isolated either from decayed wood or from the fruit bodies collected and identified by P. Vampola in forests in the Czech Republic in 1993.

Aurantioporus croceus (Pers.: Fr.) Murrill, strain 422/93, collected 7. IX. 1993 on a lying trunk of *Quercus robur* in Náměšť nad Oslavou, a nature reserve near Třebíč.

Bjerkandera adusta (Willd.: Fr.) P. Karst, strain 606/93, collected 21. X. 1993 on a lying trunk of *Populus tremula*, marches of Soběslav, Komárov, district Tábor.

Ceriporia metamorphosa (Fuckel) Ryv. et Gilbn., strain 193/93, collected 19. VI. 1993 on a lying trunk of *Quercus robur*, Ranšpurk nature reserve, Lanžhot, district Břeclav.

Daedaleopsis confragosa (Bolt.: Fr.) Schroet., strain 491/93, collected 24. IX. 1993 on a lying branch of *Fraxinus excelsior*, Polom nature reserve near Chotěboř, Horní Bradlo.

Ganoderma applanatum (Pers.) Pat., strain 164/93, collected 8. VI. 1993 on a lying trunk of *Cerasus avium*, valley of the Výrovka river, Radim, district Kolín.

Ganoderma lucidum (W. Curt.: Fr.) P. Karst., strain 530/93. collected 6. X. 1993 on a dead root of *Quercus robur*, Ranšpurk nature reserve, Lanžhot, district Břeclav.

Grifola frondosa (Dicks.: Fr.) S. F. Gray, strain 534/93, collected 6. X. 1993 on the base of a dead trunk of *Quercus robur*, Ranšpurk nature reserve, Lanžhot, district Břeclav.

Hapalopilus nidulans (Fr.) P. Karst., strain 166/93, collected 8. VI. 1993 on a lying trunk of *Prunus padus*, valley of the Výrovka river, Radim, district Kolín.

Hymenochaete tabacina (Sow.: Fr.) Lév., strain 227/93, collected 6. VII. 1993 on a dead branch of *Ribes nigrum*, garden near the village Vysoká near Jihlava.

Inonotus nidus-pici Pil., strain 189/93, collected 18. VI. 1993 in cavity of a living trunk of *Quercus cerris*, Randezvous nature reserve, Valtice near Břeclav.

Inonotus obliquus (Pers.: Fr.) Pil., strain 615/93, collected 21. X. 1993 on a living trunk of *Betula pendula*, marshes of Soběslav, Komárov, district Tábor.

Inonotus radiatus (Sow.: Fr.) P. Karst., strain 402/93, collected 7. IX. 1993 on a lying branch of *Carpinus betulus,* nature preserve near Třebíč, Náměšť nad Oslavou.

Irpex lacteus (Fr.: Fr.) Fr., strain 617/93, collected 21. 10. 1993 on a lying trunk of *Populus tremula,* Komárov, marches of Soběslav, Komárov, district Tábor.

Mycoacia sp., strain 446/93, collected on a lying trunk of *Abies alba*, Žákova hora nature reserve, Cikháj near Žďár nad Sázavou.

Pachykytospora tuberculosa (Fr.) Kotl. et Pouz., strain 505/93, collected 25. IX. 1993 on a dead branch of *Quercus* sp., nature reserve near the village Hrádek, district Hradec Králové.

Phaeolus schweinitzii (Fr.) Pat., strain 261/93, collected 17. VII. 1993 on a living trunk of *Cerasus avium,* Maršov, alley located 11 km north-west of Jihlava.

Phellinus alni (Bond.) Parm., strain 16/93, collected 21. I. 1993 on a living trunk of *Alnus incana,* valley of the Rozkošský brook, Havlíčkův Brod.

Phellinus alni (Bond.) Parm., strain 28/93, collected 21. I. 1993 on a living trunk of *Sorbus aucuparia*, field alley near the village Věž, Bezděkov, district Havlíčkův Brod.

Phellinus contiguus (Fr.) Pat., strain 141/93, collected 8. V. 1993 on a lying branch of *Sambucus racemosa,* Bradlo forest, Horní Kosov near Jihlava.

Phellinus contiguus (Fr.) Pat., strain 427/93, collected 8. IX. 1993 on a living trunk of *Fraxinus excelsior,* nature reserve Zásmuky near Kolín.

Phellinus hartigii (Allesch. et Schnabl) Bond., strain 609/93, collected 21. X. 1993 on a stump of *Picea abies,* marches of Soběslav, Komárov, district Tábor.

Phellinus hartigii (Allesch. et Schnabl) Bond., strain 249/93, collected 10. VII. 1993 on a lying trunk of *Abies alba,* Kloc nature reserve, Třešť.

Phellinus ex aff. *igniarius* (L.: Fr.) Quél., strain 619/93, collected 21. X. 1993 on a dead trunk of *Salix cinerea,* marches of Soběslav, Komárov, district Tábor.

Phellinus igniarius (L.: Fr.) Quél., strain 138a/93, collected 7. V. 1993 on a living trunk of *Salix fragilis,* valley of the Žabinec brook, Petrkov near Havlíčkův Brod.

Phellinus nigricans (Fr.) P. Karst. *sensu* Černý 1989, strain 248/93, collected 10. VII. 1993 on a lying trunk of *Fagus sylvatica,* Kloc nature reserve, Třešť.

Phellinus pilatii Černý, strain 196a/93, collected 19. VI. 1993 on a lying branch of *Populus canescens,* Ranšpurk nature reserve, Lanžhot, district Břeclav.

Phellinus pini (Thore: Fr.) A. Ames, strain 614/93, collected 21. X. 1993 on a living trunk of *Pinus uncinata,* marshes of Soběslav, Komárov, district Tábor.

Phellinus pomaceus (Pers.) Maire, strain 47/93, collected 20. VI. 1993 on a dead branch of *Prunus domestica,* Zásmuky near Kolín.

Phellinus pseudopunctatus A. David, Dequatre et Fiasson, strain 538/93, collected 6. X. 1993 on a living trunk of *Carpinus betulus*, Cahnov nature reserve, Lanžhot, district Břeclav.

Phellinus punctatus (Fr.) Pil., strain 421/93, collected 7. IX. 1993 on a lying trunk of *Carpinus betulus*, nature reserve near Třebíč, Náměšť nad Oslavou.

Phellinus robustus (P. Karst.) Bourd. et Galz., strain 204/93, collected 17. VI. 1993 by L. Hagara on a lying trunk of *Quercus* sp., forest near Adamov motel, Kbely, district Senica.

Phellinus tremulae (Bond.) Bond. et Borissov in Bond., strain 209b/93, collected 23. VI. 1993 on a lying trunk of *Populus tremula*, scrub on the Lužný pond, Vysoká near Jihlava.

Pilatoporus ibericus (Melo et Ryv.) Kotl. et Pouz., strain 190/93, collected 19. VI. 1993 on a lying trunk of *Carpinus betulus*, Ranšpurk nature reserve, Lanžhot district Břeclav.

Pleurotus ostreatus (Jacq.: Fr.) Kumm., strain 670/93, collected 7. XI. 1993 on the base of a dead trunk of *Acer pseudoplatanus*, the so-called old park on the right bank of the Jihlava river, Jihlava – Staré Hory.

Spongipellis spumeus (Sowerby: Fr.) Pat., strain 453/93, collected on a living trunk of *Acer campestre*, exhibition ground, Brno-Pisárky.

Stereum rugosum (Pers.: Fr.) Fr., strain 210/93, collected on a dead trunk of *Corylus avellana*, scrub on bank of the Lužný pond, Vysoká near Jihlava.

Trametes versicolor (L.: Fr.) Pil., strain 167/93, collected on a lying trunk of *Cerasus avium* by M. Procházková, southern slope of Vrchy hill near Pelhřimov, Vyskytná.

Tyromyces chioneus (Fr.: Fr.) P. Karst., strain 616/93 on a lying branch of *Betula pendula*, marshes of Soběslav, Komárov, district Tábor.

Screening Method

Decolorization of Poly R-478 and Remazol brilliant blue R (RBBR) was estimated on agar plates where a plug cut out of a malt extract agar culture (5 g/l malt extract Oxoid, U. K.,10 g/l glucose, 20 g/l agar, pH 4.5) of the fungus tested was inserted in a well (1 cm diameter) cut in the agar medium containing 20 g/l agar and 200 mg/l poly R-478 dye or RBBR (both Sigma, USA) and incubated at 28 °C. The growth media employed are listed below. Formation and development of a decolorized zone were observed at regular time intervals to determine the period (in days) from the inoculation to the appearance of a decolorized zone and/or a complete decolorization of the agar medium.

In addition to the decolorization capacity, also the growth of strains on the agar media was also estimated by measuring the colony diameter on an agar plate.

The following growth media were used: a mineral, low nitrogen medium (NMM) according to Tien Kirk (1988), a malt extract/glucose medium (MEG) containing per litre 5 g malt extract (Oxoid, U. K.) and 10 g glucose, pH 4.5, and a complex, high nitrogen medium (YEPG) containing 10 g glucose, 5 g peptone, 2 g yeast extract (Difco, USA), 1 g KH_2PO_4 and 0.5 g $MgSO_4 . 7H_2O$ per litre, pH 4.5.

Results and Discussion

A set of 39 strains of wood destroying Basidiomycetes studied. With the exception of two species (*Phaeolus schweinitzii* and *Pilatoporus ibericus*) causing brown rot of wood, all other strains represented white rot fungi possessing the ability to degrade lignin efficiently. In all 39 strains the ability to decolorize Poly R-478 was screened and in 33 strains of them decolorization of RBBR as well.

The two dyes tested showed a similar decolorization pattern (*cf.* Tab. 1 and 2), even though they represent chemically different types (Poly R-478 is a polymeric anthrapyridone dye with a poly(vinilamine) sulfonate backbone, RBBR is a non-polymeric vinylsulfonyl anthraquinone dye). Brown rot species did not show any decolorizing ability nor did some white rot species (*Inonotus nidus-pici, I. radiatus, Phellinus tremulae*). With other white rot species the pronounced differences were observed. Several species (*Grifola frondosa, Phellinus hartigii, P. alni, P. nigricans,*) showed only a modest decolorizing ability. Using Poly R-478 the highest number of positive decolorizing strains was detected in the MEG medium, whereas using RBBR the highest decolorization scores were observed on NMM. With the YEPG medium, using peptone as the nitrogen source which is rich in nutrients, only several strains (*Bjerkandera adusta, Inonotus obliquus, Irpex lacteus, Mycoacia* sp., *Phellinus pseudopunctatus, Pleurotus ostreatus, Trametes versicolor*) gave positive results. These strains also effectively decolorized the dyes in the other two media. This observation suggested that those strains, contrary to *Phanerochaete chrysosporium*, did not need a nutrient limit to manifest their ligninolytic activities. The decolorization of RBBR on the YEPG medium caused by *Inonotus obliquus, Phellinus pseudopunctatus* and *Trametes versicolor* occurred separated from the development of the mycelium, i.e. these fungi first produced a dense and abundant mycelium that covered the agar surface, and only then a sudden decolorization of almost the entire agar layer took place. However, most strains (typically *Aurantioporus croceus, Ceriporia metamorphosa, Hapalopilus nidulans, Phellinus* ex aff. *igniarius, Stereum rugosum, Tyromyces chioneus*) followed the *Phanerochaete chrysosporium* pattern and decolorized only on low nutrient media. This observation indicates that the general condition causing the start of ligninolitic enzyme production triggered by nutrient limitation is widely spread but cannot be applied to all white rot fungal species.

Table 1
Decolorization of agar media containing Poly R-478 dye during growth of selected wood-rot fungal strains at 28 °C on three different media

Fungal strain	Medium					
	YEPG		MEG		NMM	
	A	B	A	B	A	B
Aurantioporus croceus 422/93		-(32)	+(10)	D(15)		-(22)
Bjerkandera adusta 606/93	+(4)	D(7)	+(4)	D(6)	+(3)	D(11)
Ceriporia metamorphosa 193/93		-(32)	+(4)	D(8)	+(7)	D(13)
Daedaleopsis confragosa 491/93	++(5)	D(7)	++(5)	D(7)		-(22)
Ganoderma applanatum 164/93		-(22)		-(22)		-(22)
Ganoderma lucidum 530/93		-(32)	+(6)	D(10)	+(8)	D(14)
Grifola frondosa 534/93		-(32)	++(11)	D(24)		-(22)
Hapalopilus nidulans 166/93		-(28)	+(11)	D(28)	+(7)	D(22)
Hymenochaete tabacina 227/93		-(32)		-(32)		-(22)
Inonotus nidus-pici 189/93		-(32)		-(28)		-(22)
Inonotus obliquus 615/93		-(32)	+(17)	++(19-32)		-(22)
Inonotus radiatus 402/93		-(32)		-(32)		-(22)
Irpex lacteus 617/93	+(4)	++(5-32)	+(5)	D(9)	+(2)	D(11)
Mycoacia sp. 446/93	+(9)	+(9-32)	+(8)	+++(21-32)	+(16)	+(19-22)
Pachykytospora tuberculosa 505/93	++(13)	++(13-32)	+(5)	D(13)		-(22)
Phaeolus schweinitzii 261/93		-(32)		-(32)		-(22)
Phellinus alni 16/93		-(32)	+(15)	++(17-32)		-(22)
Phellinus alni 28/93		-(22)	+(14)	+(14-22)		-(22)
Phellinus alni 97/93		-(22)		-(22)		-(22)
Phellinus contiguus 141/93		-(22)		-(22)		-(22)
Phellinus contiguus 427/93	+(7)	+(7-22)		-(22)		-(22)
Phellinus hartigii 609/93		-(22)		-(22)		-(22)
Phellinus hartigii 249/93		-(22)		-(22)		-(22)
Phellinus ex aff. igniarius 619/93		-(22)	+(9)	D(16)	±(9)	±(9-22)
Phellinus igniarius 138A/93		-(22)	+(5)	D(14)		-(22)
Phellinus nigricans 248/93		-(22)		-(22)	++(22)	++(22)
Phellinus pilatii 196A/93		-(22)	+(17)	+++(22)	+(8)	(19-22)
Phellinus pini 614/93		-(22)		-(22)	+(8)	D(19)
Phellinus pomaceus 47/93		-(22)	+(16)	+++(22)	+(6)	+(6-22)
Phellinus pseudopunctatus 538/93	+(7)	+++(11-22)	+(7)	D(11)	+(8)	(14)
Phellinus punctatus 421/93	+(9)	+++(22)	+(7)	D(16)	+(8)	D(14)

Fungal strain	Medium					
	YEPG		MEG		NMM	
	A	B	A	B	A	B
Phellinus robustus 204/93		–(22)		–(22)		–(22)
Phellinus tremulae 209b/93		–(22)		–(22)		–(22)
Pilatoporus ibericus 190/93		–(22)		–(22)		–(22)
Pleurotus ostreatus 670/93	+(8)	+++(21–32)	+(5)	D(19)	+(5)	D(9)
Spongipellis spumeus 453/93		–(32)		–(28)		–(22)
Stereum rugosum 210/93		–(32)	+(10)	D(13)	+(7)	+++(16–22)
Trametes versicolor 167/93	+(3)	D(6)	+(2)	D(6)	+(4)	++(7–22)
Tyromyces chioneus 616/93		–(22)	+(7)	D(16)	+(6)	D(14)

Comments to Table 1
Estimation of decolorizing activity: decolorization of 1/3 of the agar plate (+), of 2/3 (++), almost total decolorization (+++), complete decolorization without any shade of red or pink (D), no decolorization (–), only slight indication of decolorization (±).
Decolorization started (A) – the corresponding growth time (days) given in parentheses; maximal degree of decolorization observed (B) – the corresponding growth time (days) needed to reach the maximal degree of decolorization or the period from maximal decolorization until the end of the experiment without any further decolorization are given in parentheses.
YEPG – yeast extract/peptone/glucose medium;
MEG – malt extract/glucose medium;
NMM – nitrogen limited medium.
Brown rot species.

Based on this screening, the strains with the most powerful decolorizing capacities were selected and they will further be used in tests of degradation of polycyclic aromatic hydrocarbons. Synthetic dyes themselves, however, represent serious environmental pollution because of their xenobiotic character, toxicity, and the fact that they have been produced and used in great amounts, mainly in the textile industry. Therefore, there is great interest in decreasing the negative impact of synthetic dyes on the environment (see Banat et al. 1996 and references therein). In this respect, the selected species with highest decolorizing capabilities also represent prospective microorganisms to be applied for the remediation of effluents and soils polluted with synthetic dyes.

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Table 2
Decolorization of agar media containing RBBR dye during growth of selected wood-rot fungal strains at 28 °C on three different media

Fungal strain	Medium					
	YEPG		MEG		NMM	
	A	B	A	B	A	B
Aurantioporus croceus 422/93		–(20)		D(20)	+(3)	D(24)
Bjerkandera adusta 606/93	+(6)	D(9)	+(3)	D(9)	+(3)	D(17)
Cerioporia metamorphosa 193/93		–(20)	++(5)	D(7)	++(5)	D(7)
Daedaleopsis confragosa 491/93	±(4)	D(21)		–(27)		–(27)
Ganoderma lucidum 530/93	+(13)	D(21)	+(3)	D(14)	+(4)	D(17)
Grifola frondosa 534/93		–(20)		–(20)		–(27)
Hapalopilus nidulans 166/93		–(20)	+(5)	D(20)	+(3)	D(17)
Hymenochaete tabacina 227/93		–(20)	+(3)	+++(20)	+(3)	+++(20)
Inonotus nidus-pici 189/93	+++(19)	D(21)	+(3)	D(24)	+(3)	D(19)
Inonotus obliquus 615/93	D(14)	D(14)	+(3)	+++(20)	+(3)	D(20)
Inonotus radiatus 402/93		–(20)		–(20)		–(20)
Irpex lacteus 617/93	+(6)	D(9)	+(3)	D(7)	++(3)	D(7)
Mycoacia sp. 446/93	+(9)	D(14)	+(4)	D(14)	+(3)	D(14)
Pachykytospora tuberculosa 505/93		–(20)		–(20)	+(3)	+++(20)
Phaeolus schweinitzii 261/93		–(20)		–(20)		–(20)
Phellinus alni 16/93		–(20)		–(20)		–(20)
Phellinus alni 28/93		–(20)	+(14)	–(20)		–(20)
Phellinus alni 97/93		–(20)		–(20)		–(20)
Phellinus hartigii 609/93		–(20)		–(20)	+(3)	D(20)
Phellinus ex aff. igniarius 138A/93		–(27)		–(20)		–(27)
Phellinus igniarius 619/93		–(20)		–(20)	+(3)	D(24)
Phellinus nigricans 248/93		–(20)		–(20)		–(20)
Phellinus pilatii 196A/93		–(20)	+(3)	D(14)	+(3)	D(14)
Phellinus pini 614/93	±(17)	+++(20)	+(11)	+++(20)	+(3)	D(20)
Phellinus pomaceus 47/93		–(19)	+(4)	D(19)	++(6)	D(19)
Phellinus pseudopunctatus 538/93	+++(17)	D(19)	+(3)	D(14)	+(3)	D(14)
Phellinus punctatus 421/93	+++(14)	D(17)	+(6)	D(16)	+(3)	D(17)
Phellinus tremulae 209b/93		–(20)		–(20)		–(20)
Pilatoporus ibericus 190/93		–(20)		–(20)		–(20)
Pleurotus ostreatus 670/93	+(8)	D(10)	+(3)	D(9)	+(3)	D(20)
Stereum rugosum 210/93		–(20)	+(10)	D(16)	+(7)	D(20)
Trametes versicolor 167/93	+++(7)	D(10)	++(3)	D(7)	++(3)	D(10)
Tyromyces chioneus 616/93	+(13)	D(27)	+(3)	D(10)	+(3)	D(14)

Comments see Table 1

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