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The Role of Mn-Dependent Peroxidase in Dye Decolorization by Static and Agitated Cultures of *Irpex lacteus*

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ABSTRACT. Dye decolorization capacity of two white-rot fungi, *Irpex lacteus* and *Phanerochaete chrysosporium*, was compared in N-limited liquid cultures. The agitated cultures showed lower ability to decolorize azo dyes Reactive Orange 16 and Naphthol Blue Black than static cultures. Similar effect was also observed with other structurally different synthetic dyes. The effect of surfactants on the decolorization process is discussed. A significant increase in the Reactive Orange 16 decolorization by the agitated *I. lacteus* cultures was observed after adding 0.1 % Tween 80, following a higher Mn-dependent peroxidase production. The *in vitro* dye decolorization using the purified enzyme proved its decolorization ability.

Abbreviations

BPB	Bromophenol Blue	MB	Methylene Blue
CSB	Chicago Sky Blue	MBTH	3-methyl-2-benzothiazolinone hydrazone
CuP	copper(II) phthalocyanine	MnP	Mn-dependent peroxidases
DB3	Disperse Blue 3	NBB	Naphthol Blue Black
DMAB	dimethylamine–borane	RBBR	Remazol Brilliant Blue R
LiP	lignin peroxidases	RO16	Reactive Orange 16

Ligninolytic fungi have been shown to degrade a wide variety of recalcitrant xenobiotics, like chlorinated organic compounds, oligocyclic (‘polycyclic’) aromatic hydrocarbons, synthetic polymers and also synthetic dyes (Paszczyński and Crawford 1995; Pointing 2001; Rabinovich *et al.* 2004). The capacity of ligninolytic fungi to degrade synthetic dyes is generally correlated with their ability to produce lignin-degrading extracellular enzymes such as LiP, MnP, or laccases (Chivukula *et al.* 1995; Heinfling *et al.* 1998; Schliephake *et al.* 2000; Saparrat and Guillén 2005).

Recently, several studies concerning the degradation of synthetic dyes by purified ligninolytic enzymes have been published. MnP isolated from *Bjerkandera* sp. BOS55 was able to decolorize azo dyes (Mielgo *et al.* 2003). Three lignin peroxidase isoenzymes obtained from *P. chrysosporium* were shown to decolorize the azo dye Crocein Orange G (Ollikka *et al.* 1998). Most of recent works have focused on the capacity of fungal laccases to decolorize synthetic dyes (Schliephake *et al.* 2000; Soares *et al.* 2001; Campos *et al.* 2001; Claus *et al.* 2002).

Though the involvement of ligninolytic enzymes in dye degradation process has been proposed due to the capability of purified enzyme activities to decolorize dyes *in vitro*, the dye degradation mechanisms used by white-rot fungi still remain unclear. The importance of the role of ligninolytic enzymes in the dye degradation process in fungal cultures under *in vivo* conditions is disputed since other processes can also be responsible for the decolorization capacity: dye sorption to the fungal biomass and the participation of activated oxygen species (Wang and Yu 1998; Hammel *et al.* 2002).

Here we compared the capacity of two fungal strains, *Irpex lacteus* and *Phanerochaete chrysosporium*, to decolorize synthetic azo dyes. *I. lacteus* was shown to produce all three main ligninolytic activities (LiP, MnP, laccases) in liquid cultures (Kasinath *et al.* 2003; Novotný *et al.* 2004). The involvement of MnP in dye decolorization could be demonstrated (Shin 2004; Shin *et al.* 2005); however, decolorization rates did not correlate with the MnP levels detected in the fungal cultures. We showed that a nonionic surfactant Tween 80 enhanced MnP production in agitated *I. lacteus* cultures and simultaneously improved dye decolorization by these cultures. An enzymic study using a purified MnP from *I. lacteus* proved the dye decolorization ability of the enzyme.

MATERIALS AND METHODS

Organisms. The fungal strains *P. chrysosporium* D237 and *I. lacteus* 917/93 were obtained from the culture collection CCBAS (*Academy of Sciences of the Czech Republic*, Prague). The fungi were grown on malt extract–glucose agar plates at 28 °C for 7 d and then maintained at 4 °C until use.
Dyes. The dyes used were:

	λ_{max}, nm		λ_{max}, nm
BPB	589	MB	665
CSB	618	NBB	618
CuP	694	RBBR	592
DB3	643	RO16	494

All dyes were obtained from *Sigma* (USA).

Culture conditions and dye decolorization. Mycelium from a malt-agar plate was used to inoculate 30 mL of N-limited mineral medium (Tien and Kirk 1988) in a 250-mL Erlenmeyer flask. After a 7-d incubation at 28 °C, the cultures were homogenized and 1 mL volumes of the homogenate were transfered to 100-mL Erlenmeyer flasks containing 10 mL of culture medium and 150 mg/L of the dye. The flasks were incubated on a reciprocal shaker (2 Hz; agitated cultures) at 28 °C. The dye decolorization was measured spectrophotometrically at the corresponding λ_{max}. All experiments were done in triplicates. Uninoculated flasks served as controls (abiotic decolorization).

To study the effect of nonionic surfactants on dye decolorization, 5 surfactants were used: Brij 30 (*Serva*, Germany), Tergitol NP40 (*Sigma*, USA), Triton X-100 (*BDH Chemical*, UK), Tween 40 (*Loba*, Germany), and Tween 80 (*Serva*, Germany). The compounds (final concentration 0.1 %) were added to 6-d-old cultures of *I. lacteus* grown in the absence of any dye. The RO16 dye was added at a final concentration of 150 mg/L and decolorization was measured for the next 25 h. Autoclaved cultures served as the controls (sorption of the dye on fungal mycelium). Uninoculated flasks served to evaluate the abiotic decolorization. In case of Tween 80, 100 mg/L of cycloheximide was added to 1 set of cultures to inhibit protein synthesis.

Enzyme assays. Laccase activity was determined by oxidation of 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) according to Bourbonnais and Paice (1990). MnP and LiP activity was determined with DMAB or MBTH and veratryl alcohol, respectively (Vyas *et al*. 1994) and was expressed in katals (1 kat = 1 mol of the enzyme substrate oxidized per s).

Dye decolorization by purified MnP. MnP was isolated from *I. lacteus* cultures (Rothschild *et al.* 2002). Briefly, the culture filtrate was frozen to remove polysaccharides by precipitation and concentrated by ultrafiltration with a 10-kDa cut-off membrane. The crude enzyme mixture was further purified (*1*) on Q Sepharose and then (*2*) on Mono Q ion exchangers, resulting in separation of isoforms with distinct p*I* (Nikku-Paavola *et al*., *unpublished results*).

A mixture of all isoforms after the 1st chromatographical step was used for the dye decolorization. The reaction system contained 0.1 mol/L succinate–lactate buffer (pH 4.5), 0.1 mmol/L $MnSO_4$, 0.1 mmol/L H_2O_2, 25 mg/L of the dye, 0.1 % Tween 80 and 80 pkat of MnP in a final volume of 200 μL. The dye concentration was measured spectrophotometrically at appropriate time intervals. The enzyme was boiled for 10 min and used as the control.

RESULTS AND DISCUSSION

The capacity of two white-rot fungi, *P. chrysosporium* and *I. lacteus*, to decolorize synthetic dyes in liquid cultures was compared. The fungi were able to decolorize all dyes but the decolorization of the thiazine dye MB by both fungi was rather limited (Table I). A difference in the RO16 and CuP decolorization capacity was observed between static and agitated fungal cultures; this difference was typical of *P. chrysosporium* where it was detected for all azo dyes as well as with RBBR, CuP and BPB. Such a difference was not observed with DB3 and MB, dyes whose degradability was rather low. In contrast to our results, Swamy and Ramsay (1999) working with *P. chrysosporium*, and *Bjerkandera* sp. BOS55 with *Trametes versicolor*, reported that agitation resulted in a greater decolorization of different azo dyes compared to static cultures.

The effect of 5 nonionic surfactants on dye decolorization was ascertained in both static and agitated cultures of *I. lacteus* decolorizing RO16. Most of the surfactants had a positive effect on decolorization by agitated cultures but, in contrast, the decolorization by static cultures was not enhanced or was even slowed down (Fig. 1). For a number of dyes, the addition of 0.1 % Tween 80 improved the otherwise limited

Table I. Decolorization (%) of synthetic dyes by static and agitated cultures of *P. chrysosporium* and *I. lacteus*

Dye	*P. chrysosporium*		*I. lacteus*	
	static	agitated	static	agitated
RO16	88 ± 2.1	30.3 ± 1.2	92 ± 0.5	69 ± 6.3
CSB	98 ± 0.2	27.0 ± 2.3	95 ± 0.9	93 ± 0.5
NBB	71 ± 0.5	7.0 ± 0.3	95 ± 0.2	92 ± 0.1
DB3	32.5 ± 2.7	44 ± 8.8	75 ± 6.0	91 ± 2.3
RBBR	97 ± 1.4	49 ± 1.7	99.7 ± 0.5	97 ± 1.2
MB	14.2 ± 6.4	22.2 ± 2.4	26.0 ± 1.1	12.0 ± 7.3
CuP	94 ± 2.9	24.2 ± 1.5	86 ± 6.7	15.3 ± 6.7
BPB	99 ± 0.3	22.7 ± 1.2	99 ± 0.3	93 ± 4.3

decolorization capacity of static and agitated cultures of *I. lacteus* and *P. chrysosporium* (Table II). In contrast, its addition to a decolorizing culture where no limitation was observed (*e.g.*, *I. lacteus* decolorizing RBBR) had no influence on the decolorization rate. Nonionic surfactants are known to enhance the biodegra-

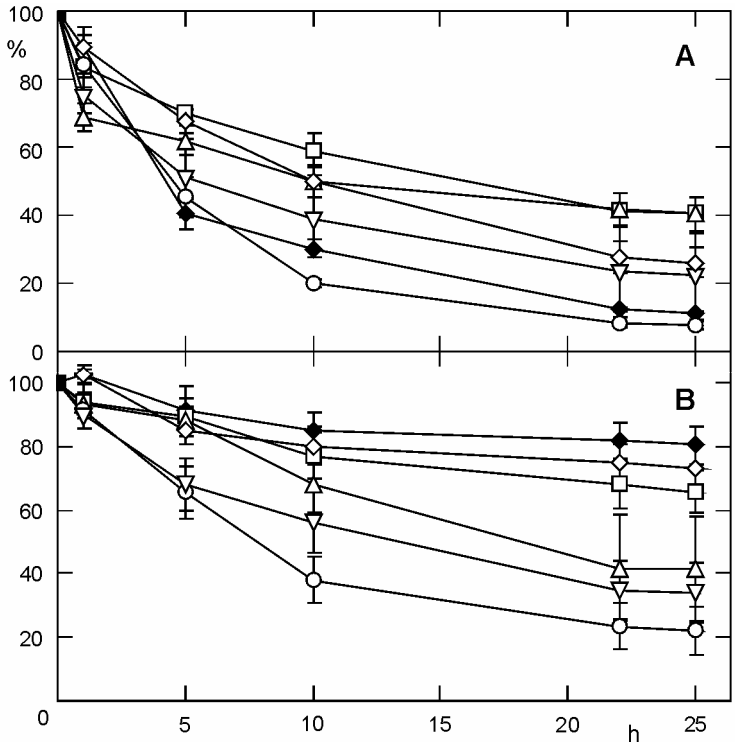

Fig. 1. Decolorization of RO16 (initial concentration 150 mg/L) by 6-d-old static (**A**) and agitated (**B**) cultures of *I. lacteus* in the presence of non-ionic surfactants: *closed rhombs* – without the addition, *triangles* – 0.1 % Triton X100, *open rhombs* – 0.1 % Tween 40, *squares* – 0.1 % Brij 30, *inverted triangles* – 0.1 % Tergitol NP40, *circles* – 0.1 % Tween 80.

Table II. Dye decolorization maxima (%) observed in cultures of *I. lacteus* and *P. chrysosporium* without (–) Tween 80 and in the presence (+) of 0.1 % Tween 80[a]

Dye	*I. lacteus*				*P. chrysosporium*			
	static		agitated		static		agitated	
	(–)	(+)	(–)	(+)	(–)	(+)	(–)	(+)
RO16	92 ± 0.5	98 ± 1.3	69 ± 6.3	99 ± 1.0	90 ± 2.8	79 ± 2.8	49 ± 3.2	93 ± 0.9
CuP	86 ± 7.0	98 ± 0.3	15.3 ± 6.0	98 ± 1.5	96 ± 2.0	66 ± 3.3	29 ± 7.2	96 ± 1.0
RBBR	99 ± 0.5	96 ± 1.0	96 ± 1.2	98 ± 0.3	99 ± 0.1	69 ± 3.8	14.0 ± 9.4	85 ± 2.0
CSB	–	–	–	–	99 ± 0.2	57 ± 6.0	68 ± 1.2	49 ± 7.0
NBB	–	–	–	–	86 ± 1.8	63 ± 1.4	7.0 ± 0.3	32 ± 3.4
BPB	–	–	–	–	99 ± 0.1	99 ± 0.1	19.5 ± 1.0	48 ± 1.0

[a]The initial dye concentration was 150 mg/L.

dation of xenobiotics, such as oligocyclic aromates, by increasing their bioavailability (Zheng and Obbard 2001). Kim and Song (2003) reported a positive influence of Tween 80 on degradation of 2,4,6-trinitrotoluene by *I. lacteus*. Tween 80 was shown to improve biodegradation capability of *P. chrysosporium* by enhancing the production of MnP by this fungus (Couto *et al.* 2000; Urek and Pazarlioglu 2005).

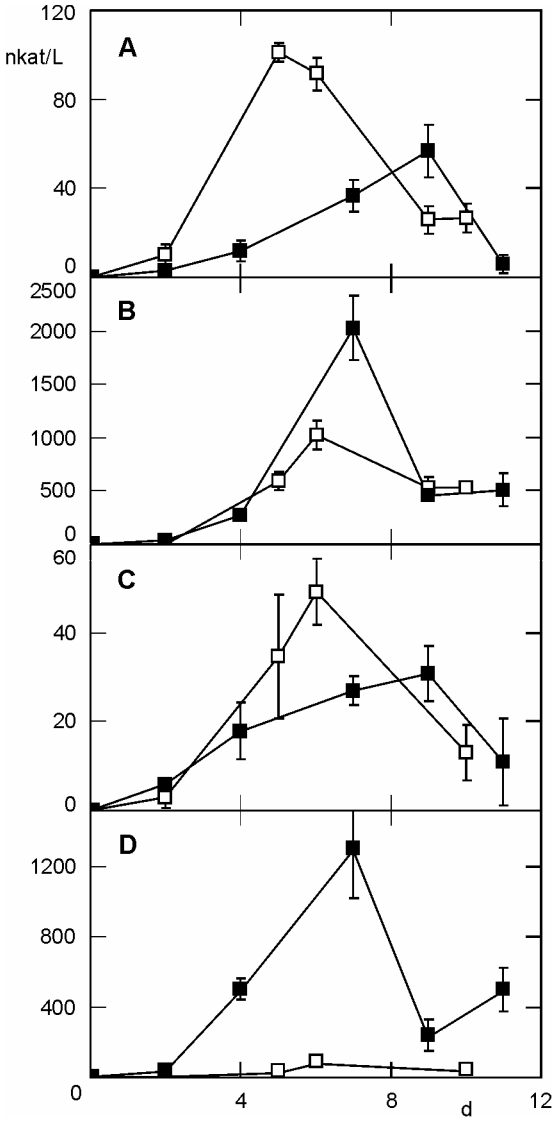

Fig. 2. Enzyme activities produced by *I. lacteus* without (*empty symbols*) or with 0.1 % Tween 80 (*closed symbols*); **A** – laccase, static cultures; **B** – MnP, static cultures; **C** – laccase, agitated cultures; **D** – MnP, agitated cultures.

The dye decolorization by fungal cultures is often correlated to ligninolytic enzyme activities (Pointing 2001; Selvam *et al.* 2003). We detected laccase and MnP activity in cultures during the decolorization process (Fig. 2) with significantly higher level of MnP present in static cultures, compared with the shaken ones, suggesting that MnP could play an important role in the dye decolorization. No LiP was found in either type of culture. The addition of Tween 80 led to up to 10× higher MnP level in shaken cultures (compared with the control cultures). In contrast, the laccase activity secreted to the culture liquid was rather low with or without Tween 80, its levels amounting to a maximum of 0.1 μkat/L.

The enhancement of the capacity of *I. lacteus* agitated cultures to decolorize RO16 could be attributed to a higher MnP activity produced by the Tween-treated cultures. To confirm this the dye decolorization was measured in the presence of 100 mg/L cycloheximide (a protein synthesis inhibitor; we used it in a concentration much higher than was used for inhibition of LiP and MnP synthesis in *P. chrysosporium* by Pease and Tien 1992) (Fig. 3). The addition of cycloheximide to Tween-containing, agitated cultures of *I. lacteus* eliminated the effect of Tween 80 on decolorization; this demonstrates that the enhancement of decolorizing capacity by Tween 80 required *de novo* MnP synthesis.

Our results are in accordance with those of Shin (2004) who suggested a correlation between the ability of *I. lacteus* static cultures to decolorize a textile industry wastewater more effectively than agitated cultures and a higher production of MnP and a non-specific peroxidase in the former cultures. Shin *et al*. (2005) isolated MnP-like enzyme from liquid cultures of *I. lacteus* and proved its ability to degrade various textile dyes. However, a direct correlation of MnP activity with the dye decolorization rate was not demonstrated (Maximo and Costa-Ferreira 2004; Shin 2004). We demon-

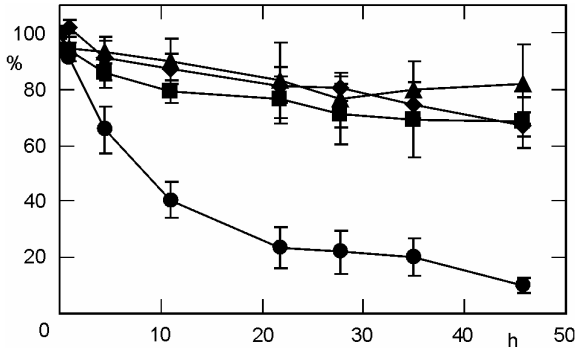

Fig. 3. Decolorization of RO16 (added to 6-d-old cultures at an initial concentration of 150 mg/L) by agitated cultures of *I. lacteus*; *rhombs* – nontreated cultures, *circles* – treated with 0.1 % Tween 80, *squares* – treated with 0.1 % Tween 80 and 0.1 mg/mL cycloheximide, *triangles* – treated with 0.1 mg/mL cycloheximide.

strated that Tween 80 really stimulated MnP production by the agitated fungal cultures and so improved the dye decolorization by these cultures.

Moen and Hammel (1994) and Harazono *et al.* (2003) reported that unsaturated fatty acids (also present in Tween 80 as oleate) can be peroxidized by MnP, and the oxidants so generated could participate in organopollutant degradation by fungal cultures. We found that Tween 40 containing saturated palmitic acid was also able to stimulate the decolorization of RO16 by agitated cultures of *I. lacteus* (Fig. 1). The observed effect was significantly lower compared to Tween 80, which would suggest that the effect of the latter surfactant on decolorization could partially be due to the radical mechanism.

MnP from *I. lacteus* was able to decolorize 4 different synthetic dyes *in vitro* (Fig. 4). An amount representing 24.1 ± 0.5 % of the initial RO16 concentration of 25 mg/L was removed from the reaction mixture by 80 pkat of the enzyme within 4 h, showing decolorization efficiency comparable with that of 6-d-old static fungal cultures. The decolorization rate, however, was not affected by the addition of Tween 80 which indicated that the surfactant or its oxidation products did not participate in dye decolorization. The enzyme at the same concentration was also able to decolorize 95 ± 0.1 % of RBBR, 88 ± 1.2 % of BPB, and 39.5 ± 2.8 % of CuP within the same time showing decolorization capacity comparable with other purified ligninolytic enzymes isolated from various white-rot fungi (Young and Yu 1997; Ollikka *et al.* 1998; Heinfling *et al.* 1998; Soares *et al.* 2001; Claus *et al.* 2002; Moldes *et al.* 2003; Mielgo *et al.* 2003).

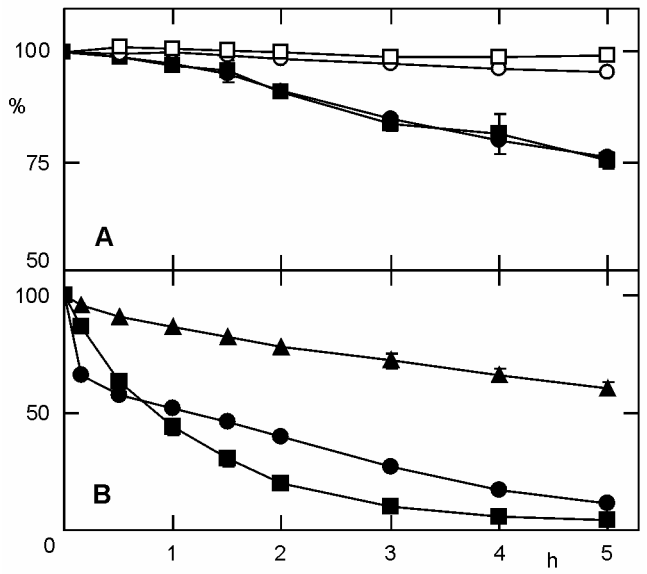

Fig. 4. Decolorization of RO16 (initial concentration 25 mg/L) by a purified MnP from *I. lacteus*; **A** – RO16 alone, *closed squares* – by MnP, *closed circles* – by MnP and 0.1 % Tween 80, *open squares* – by heat inactivated MnP (100 °C, 10 min); *open circles* – by heat inactivated MnP and of 0.1 % Tween 80; **B** – decolorization of synthetic dyes: *triangles* – CuP, *squares* – BPB; *circles* – RBBR.

Our results demonstrate the ability of MnP from *I. lacteus* to decolorize structurally diverse synthetic dyes, which strongly suggests the implication of this enzyme in decolorization cultures *in vivo*. Evidence was brought that a low MnP production was responsible for the limitation of decolorization capacity of agitated cultures since the decolorization could be stimulated by the addition of Tween 80 resulting in a stimulation of MnP production.

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