

SCREENING OF FUNGAL STRAINS FOR REMEDIATION OF WATER AND SOIL CONTAMINATED WITH SYNTHETIC DYES

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Abstract

Using Remazol Brilliant Blue R (RBBR), strains of terrestrial white rot (WRF) and marine fungi (MF) were screened for efficient decolorization. Dye degradation potential of selected strains was studied with chemically different dyes (azo, anthraquinone, heterocyclic, triphenylmethane). *Irpex lacteus* and *Pleurotus ostreatus* (WRF) efficiently degraded dyes from all groups whereas less efficient and selective degradations were observed with *Dactylospora haliotrepha* and *Aspergillus ustus* (MF). Seawater salinity often reduced decolorization efficiency of WRF but increased decolorization ability of MF. In soil *I. lacteus* removed 77 % of RBBR used at 150 µg/g within 6 weeks. The work presents fungi as suitable candidates to be applied to re-mediation of dye-contaminated water and soil.

1. Introduction

More than 10 000 commercially available dyes representing a total of 700 000 tons of dyestuff are produced worldwide annually, 10 % of which enters industrial effluents. Many dyes are resistant to conventional wastewater treatment (Shaul et al. 1991) and, therefore, the need exists for development of new processes for treating those effluents. Bioremediation by fungi offers an alternative since some ligninolytic species are efficient dye degraders (Paszczynski and Crawford 1995; Banat et al. 1996; etc.).

The biodegradation potential of the majority of ligninolytic fungal species existing in nature as well as of marine fungal species is poorly known (Paszczynski and Crawford 1995; Raghukumar et al. 1996; Swamy and Ramsay 1999). The purpose of our study was to select strains of terrestrial white rot and marine fungi that would effectively decolorize recalcitrant dyes and would be applicable to bioremediation of dye-contaminated water and soil. Since in some countries the pollution with dyes affects large areas of marine coast environment, the effect of seawater salinity on decolorization capacities of the selected fungal strains was documented.

2. Material and Methods

2.1. MICROORGANISMS

The following strains of terrestrial fungi were used: *Bjerkandera adusta* 606/93; *Ceriporia metamorphosa* 193/93; *Daedaleopsis confragosa* 491/93; *Ganoderma lucidum* 530/93; *Irpex lacteus* 617/93; *Mycoacia* sp. 446/93; *Pachykytospora tuberculosa* 505/93; *Phellinus pseudopunctatus* 538/93; *Phellinus punctatus* 421/93; *Pleurotus ostreatus* 670/93; *Stereum rugosum* 210/93; *Trametes versicolor* 167/93 and *Tyromyces chioneus* 616/93.

The following marine micromycetes were used: *Anguillospora longissima* M234; *Aniptodera mangrovei* M235; *Aspergillus ustus* M224; *Banhegia setispora* M236; *Bathyascus* sp. M237; *Corollospora pulchella* M217; *Dactylospora haliotrepha* M240; *Flagellospora curvula* M241; *Hypoxylon oceanicum* M244; *Lulworthia grandispora* M245; *Paraliomyces lentiferus* M248; and *Zalerion* sp. M249.

All strains were maintained on MEG agar slants at 4 °C.

2.2. SCREENING ON AGAR PLATES AND BIODEGRADATION OF DYES

The following agar (20 g/L; Oxoid, U.K.) media were used, containing (per liter): MEG pH 4.5, 5 g malt extract and 10 g glucose; YEPG pH 4.5, 2 g yeast extract, 5 g peptone, 10 g glucose, 1 g KH_2PO_4 and 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; low nitrogen mineral medium (Tien and Kirk 1988) pH 4.5 (LNMM); and GPY pH 6.0, 1 g glucose, 0.5 g peptone and 0.1 yeast extract. Artificial seawater (3 % salinity, W/V) was according to Lorenz and Molitoris (1997). Sterilization was done by autoclaving (120 °C, 20 min).

Decolorization of dyes was evaluated after inoculation of the dye-containing (200 µg/mL) agar plate with a mycelium-covered disc removed from a fresh MEG culture and subsequent incubation at the appropriate temperature. Decolorization and growth were read in time. Control plates without fungal inoculation were always included in the experiments. All cultures were run in duplicate.

The following dyes were used: Methyl Red (monoazo; Sigma, Germany), Methyl Orange (monoazo; Lachema, Czech Republic), Reactive Orange 16 (monoazo; Aldrich, USA), Congo Red (disazo; Merck, Germany), Reactive Black 5 (disazo; Aldrich, Germany), Naphthol Blue Black (disazo; Aldrich, USA), Remazol Brilliant Blue R (RBBR) (anthraquinone; Sigma, USA), Disperse Blue 3 (anthraquinone; Aldrich, USA), Fluorescein (heterocyclic; Lachema, Czech Republic), Methylene Blue (heterocyclic thiazine; Merck, Germany), Bromophenol Blue (triphenylmethane; Lachema, Czech Republic).

2.3. SCREENING WITH RBBR-CELLULOSE

Two-layer agar medium based on artificial seawater (Lorenz and Molitoris 1997) containing (per liter) 0.5 g peptone, 0.1 g yeast extract and 16 g agar was used, the upper

layer contained 5 g RBBR-cellulose per liter. The medium was inoculated on the top with a mycelium-covered disc removed from a fresh fungal culture and incubated at for 7 weeks to detect cellulase activity and decolorization of RBBR.

2.4. DYE DEGRADATION IN SOIL

An amount of 8 g of dry tyndalized brown soil (particles <2 mm; native humidity 5.4 %, pH 6.5, organic carbon 4.7 % glucose equivalent) was used in 100-mL Erlenmeyer flasks. The soil was homogenously spiked with RBBR (final concentration 150 µg/g; moisture content 20 %, W/W). Freshly prepared, straw-grown *I. lacteus* (8.0 g) was mixed with the RBBR-spiked soil. Controls contained either heat-killed inoculum or sterilized straw without the fungus. All flasks were incubated at 28°C. The contaminated soil was extracted and analyzed on days 0, 14, 28, 35, 42 and 49 after inoculation. All live samples and controls were extracted and analyzed in triplicate.

RBBR was extracted from the spiked soil using a multisolvent system made up of chloroform, methanol and distilled water (1:1:1, V/V). Each solvent was added separately in the above order, followed by mixing and vigorous shaking of the soil sample. The soil sample was sonicated (15 min) and filtered, the filtrate was collected and chloroform removed using a separation funnel. After centrifugation the filtrate was placed in an open glass Petri dish and the solvents evaporated at 100 °C for 6-8 h. The residues were redissolved in distilled water and centrifuged. After dilution, absorbance was read at 578 nm using a Perkin-Elmer λ 11 UV-VIS spectrophotometer.

3. Results and Discussion

3.1. SCREENING OF TERRESTRIAL AND MARINE FUNGI

A broader preselection (not shown) using RBBR of terrestrial, wood-rotting fungal strains capable of efficient decolorization of dyes involved more than 100 strains collected in forests of central Europe or obtained from the Culture Collection of Basidiomycetes, Institute of Microbiology, Prague, to cover the following genera: *Abortiporus*, *Agaricus*, *Anthrodia*, *Aspergillus*, *Aurantioporus*, *Aureobasidium*, *Bjerkandera*, *Ceriporia*, *Cerrena*, *Corioloopsis*, *Daedalea*, *Daedaleopsis*, *Dictyostelium*, *Flammulina*, *Fomes*, *Fomitopsis*, *Ganoderma*, *Gleophyllum*, *Grifola*, *Hapalopilus*, *Hymenochaete*, *Inonotus*, *Irpex*, *Ischnoderma*, *Laricifomes*, *Lentinus*, *Leucoagaricus*, *Mycoacia*, *Pachykytospora*, *Panus*, *Phaeolus*, *Phallus*, *Phanerochaete*, *Phellinus*, *Phlebia*, *Phlebiopsis*, *Phlyctochytrium*, *Pycnoporus*, *Pilatoporus*, *Pleurotus*, *Podospora*, *Schizophyllum*, *Sphaerobolus*, *Spongipellis*, *Stereum*, *Trametes*, *Tyromyces*.

Table 1 documents that the most efficient of the preselected RBBR degraders were able to grow well in the presence of the dye and decolorized the plate completely within 10 days. The decolorization efficiency was tested in mineral and complex media conditions. The content of nitrogen source, known to control the production of ligninolytic peroxidases in white rot fungi (Kaal et al. 1995), was from 0.002-0.003 % (LNMM, MEG) to 0.07 % (YEFG). Some strains decolorized RBBR rapidly under

broad nutritive conditions (*I. lacteus*, *T. versicolor*) others preferred specific conditions to a different degree (*C. metamorphosa*, *B. adusta*, *P. ostreatus*) (Table 1).

I. lacteus and *P. ostreatus* efficiently colonized sterile soil by exploratory mycelium whereas *T. versicolor* was capable of only a partial colonization and *B. adusta* did not grow into the soil at all (not shown). Therefore, *I. lacteus* and *P. ostreatus* were chosen for the comparison of their decolorizing capacities with those of marine fungi.

Screening of dye-degrading strains of marine fungi originating from the Botanical Institute, University of Regensburg with RBBR-cellulose was carried out under the conditions of both freshwater and seawater salinity (not shown). Only two of the fungi, *A. ustus* and *D. haliotrepha*, were able to decolorize the plates at both salinities and were therefore used in the comparative study.

TABLE 1. Screening of wood-rotting fungal strains on RBBR-containing agar media.

Fungus	Medium ^{1,2}					
	YEPG		MEG		LNMM	
	A ³	B ⁴	A ³	B ⁴	A ³	B ⁴
<i>Bjerkandera adusta</i>	7	9	10	9	P	17
<i>Cerioporia metamorphosa</i>	6	-	7	7	7	7
<i>Daedaleopsis confragosa</i>	P	21	P	-	P	-
<i>Ganoderma lucidum</i>	P	21	12	14	12	17
<i>Irpex lacteus</i>	5	9	7	7	9	7
<i>Mycocacia</i> sp.	14	14	14	14	14	14
<i>Pachykytospora tuberculosa</i>	P	-	P	-	P	P
<i>Phellinus pseudopunctatus</i>	19	19	17	14	14	14
<i>Phellinus punctatus</i>	17	17	17	16	P	17
<i>Pleurotus ostreatus</i>	10	10	10	9	14	20
<i>Siereum rugosum</i>	17	-	17	16	P	20
<i>Trametes versicolor</i>	9	10	7	7	P	10
<i>Tyromyces chioneus</i>	P	27	10	10	14	14

¹ Culture medium contained 200 mg RBBR per litre; ² cultures were incubated at 28°C for 27 days;

³ A - growth expressed as a number of days necessary for colonization of the whole plate; P, only a part of the plate surface was colonized within 27 days; ⁴ B - decolorization expressed as a number of days necessary for complete decolorization of the plate; P, partial decolorization after 27 weeks; -, no decolorization.

3.2. DECOLORIZATION OF VARIOUS DYES

In order to compare dye degradation capacities of the selected representatives of terrestrial ligninolytic basidiomycetes and marine micromycetes, a set of compounds belonging to four major groups of recalcitrant dyes was used (Table 2). Terrestrial fungi were superior to marine fungi in having the ability to decolorize all types of dyes. *I. lacteus* and *P. ostreatus* thus belong to those few white rot fungal species, such as *T. versicolor*, *P. chrysosporium* and *Bjerkandera* sp., that are capable of efficient decolorization of a broad spectrum of chemically different dyes (Knapp *et al.* 1995; Swamy and Ramsay 1999). The results obtained with *P. ostreatus* (Table 2) confirmed those of Knapp *et al.* (1995) who demonstrated the ability of their natural isolate of this fungal species to decolorize high concentrations of various azo-, anthraquinone-, Cu-phthalocyanine- and triphenylmethane dyes. *D. haliotrepha* was able to attack all dyes except for Reactive Orange 16 and Disperse Blue 3, but many dyes were decolorized

only partially. *A. ustus* was able to attack only less than a half of the dyes tested and the decolorization was mostly incomplete (Table 2). We cannot compare our data obtained with the two latter fungi with those of other studies since the ability of marine fungi to degrade synthetic dyes is mostly unknown (Raghukumar et al. 1996).

Decolorization by terrestrial fungi of some dyes was slowed down or even completely blocked by seawater salinity (Table 2). In some cases, however, the decolorization was not affected by the salinity or even a positive effect could be observed (Congo Red). Different effects of the salinity in *I. lacteus* and *P. ostreatus* were observed in the case of degradation of Disperse Blue 3, Fluorescein and Bromophenol Blue (Table 2). Reasons for such diverse salinity effects on decolorization of various dyes by white-rot fungi are unknown.

As far as the marine fungi were concerned, seawater salinity enabled them to degrade disazo-, anthraquinone-, heterocyclic- and triphenylmethane dyes (Table 2). On the other hand, Methyl Red and, in the case of *D. haliotrepha*, also Congo Red, could be decolorized only if deionized water was used to prepare the medium.

In general, the results confirm the hypothesis of a lower degree of salinity stress (cf. Capone and Bauer 1992) in the marine fungi compared to the terrestrial ones. However, the decolorization by *I. lacteus* and *P. ostreatus* under seawater salinity conditions shows that even under those stressing conditions these fungi can use their biochemical machinery for dye degradation.

3.3. DYE DEGRADATION IN SOIL

The ability of *I. lacteus* to degrade dyes in soil was tested using RBBR at 150 µg/g

TABLE 2. Decolorization of synthetic dyes by terrestrial and marine fungi on agar medium containing deionized water or artificial seawater.

Dye	Decolorization efficiency ¹							
	<i>Irpex lacteus</i>		<i>Pleurotus ostreatus</i>		<i>Dactylospora hal.</i>		<i>Aspergillus ustus</i>	
	DW ²	SW ³	DW ²	SW ³	DW ²	SW ³	DW ²	SW ³
Methyl Red	9	29	15	P	10	-	17	-
Methyl Orange	9	11	10	25	12	P	-	-
Reactive Orange 16	10	14	15	29	-	-	-	-
Congo Red	20	14	22	14	P	-	-	-
Reactive Black 5	11	16	13	14	-	25	-	P
Naphtol Blue Black	27	25	17	16	-	38	-	P
RBBR	10	10	13	14	-	P	-	P
Disperse Blue 3	17	16	12	38	-	-	-	P
Fluorescein	15	11	13	21	-	P	-	-
Methylene Blue	22	25	20	-	-	P	-	-
Bromophenol Blue	12	12	27	14	-	P	-	-

¹ Cultures were incubated at 22°C for 6 weeks. Decolorization is expressed as a number of days necessary for complete decolorization of the plate; P, partial decolorization after 6 weeks; -, no decolorization; ² DW, GPY medium based on deionized water, ³ SW, GPY medium based on artificial seawater.

as the model dye. The tyndalized, RBBR-spiked soil was inoculated by mixing with freshly prepared, straw-grown fungal inoculum and the dye removal was detected in time. After 6 weeks, 77 % of the original amount of RBBR was removed, the

disappearance being linear at a constant rate of 2.8 µg RBBR/g soil per day (not shown). The results suggest that recalcitrant dyes, like other organopollutants, can be removed from the soil environment by the action of ligninolytic fungi (cf. Andersson and Henrysson 1996; Novotný et al. 1999; etc.).

The study demonstrates that effective dye degraders applicable to remediation of freshwater, seawater and soil environments contaminated with recalcitrant dyes can be screened from among unknown strains of terrestrial and marine fungal organisms.

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