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Degradative Properties of Two Newly Isolated Strains of the Ascomycetes *Fusarium oxysporum* and *Lecanicillium aphanocladii*

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Abstract

Two ascomycete strains were isolated from creosote-contaminated railway sleeper wood. By using a polyphasic approach combining morpho-physiological observations of colonies with molecular tools, the strains were identified as *Fusarium oxysporum* Schltdl. (IBPPM 543, MUT 4558; GenBank accession no. MG593980) and *Lecanicillium aphanocladii* Zare & W. Gams (IBPPM 542, MUT 242; GenBank accession no. MG593981). Both strains degraded hazardous pollutants, including polycyclic aromatic hydrocarbons, anthraquinone-type dyes, and oil. Oil was better degraded by *F. oxysporum*, but the aromatic compounds were better degraded by *L. aphanocladii*. With both strains, the degradation products of anthracene, phenanthrene, and fluorene were 9,10-anthraquinone, 9,10-phenanthrenequinone, and 9-fluorenone, respectively. During pollutant degradation, *F. oxysporum* and *L. aphanocladii* produced an emulsifying compound(s). Both fungi produced extracellular Mn-peroxidases, enzymes possibly involved in the fungal degradation of the pollutants. This is the first report on the ability of *L. aphanocladii* to degrade four-ring PAHs, anthraquinone-type dyes, and oil, with the simultaneous production of an extracellular Mn-peroxidase.

Keywords *Fusarium oxysporum* ● *Lecanicillium aphanocladii* ● degradation ● pollutants ● Mn-peroxidase

Introduction

Ascomycetes are a large group of higher fungi that are widely distributed in the world. They inhabit various ecological niches, are highly adaptable, and have different types of relationships with other organisms – from mutualism to parasitism. Many ascomycetes are both useful and harmful to humans, which makes them important for research and use in biotechnology. The degradative properties of ascomycetes are well-known also (Harms 2011; Aranda 2016).

Fusarium fungi are diverse and are omnipresent in nature. Except the well-known *Fusarium* pathogens, most species and strains are saprotrophic and live in soil, utilizing lignin, complex carbohydrates, and plant litter as their carbon sources. Some strains form a symbiosis with plants, protect them from diseases (Gordon et al. 1989; Lemanceau et al. 1993), and even promote host growth, increasing plant biomass and indirectly increasing the efficiency of phytoextraction (Zhang et al. 2012).

Fusarium fungi are also implicated in the degradation of pollutants. For example, *F. oxysporum* significantly reduced the concentration of oil (Fariba et al. 2010) and degraded and mineralized anthracene, phenanthrene, and pyrene (Jacques et al. 2008). Two *F. solani* strains (H30 and H50) and one *F. oxysporum*

strain (H80) converted glyphosate to unidentified metabolites (Krzysko-Lupicka and Sudol 2008). In combination with *Rhodococcus erythropolis*, *F. solani* effectively degraded benzo[α]pyrene, toluene, and formaldehyde (Morales et al. 2017); and in combination with *Arthrobacter oxydans* (included in microcosms with alfalfa), *F. solani* significantly reduced the concentrations of phenanthrene, pyrene, and dibenz[a,h]anthracene (Thion et al. 2013).

Whereas *Fusarium* fungi have been studied for more than 100 years, the genus *Lecanicillium* was introduced relatively recently (Zare and Gams 2001). The study of newly isolated *Lecanicillium* strains relates primarily to their entomopathogenic properties (Ali et al. 2017). However, some *Lecanicillium* species are biotechnologically beneficial. For example, *L. muscarium* is a potent producer of extracellular cold-resistant, chitin-hydrolyzing enzymes (Fenice 2016); and *L. aphanocladii* produces the pigments osporine, orivactaine, and dihydricricodimerol (Souza et al. 2016). *L. aphanocladii* was formerly known commonly as *Verticillium lecanii* (Zimmerman) Viegas, a producer of verticillin (Gingina et al. 1990). The degradative properties of *Lecanicillium* have been poorly studied. There are data that *L. saksenae* is a good decomposer of the pesticide pendimethalin (Pinto et al. 2012) and that *V. lecanii* is a decomposer of 2,4-dichlorophenol and 2,4-dichlorophenoxyacetic acid (Vroumsia et al. 1999) and of anthracene (Krivobok et al. 1998).

The degradative activities of Ascomycota are often attributed to their extracellular enzyme systems. These can be similar to the ligninolytic systems of basidiomycetes, which include lignin peroxidase, Mn-peroxidase, and laccase. These enzymes catalyze the key steps of the degradation of lignin and a wide range of aromatic pollutants (Wong 2009; Kadri et al. 2017). The ascomycete enzymes have received much study. *Fusarium* species have ligninolytic enzymes such as Mn-peroxidase, lignin peroxidase, and laccase, which are involved in stress reactions and in the degradation of lignocelluloses (Obruca et al. 2012). The laccases of ascomycetes have been well described. The role of laccase in fungal pathogenesis has been confirmed in numerous studies (Kwiatos et al. 2015), and the laccase genes of *F. oxysporum* were functionally analyzed (Cañero and Roncero 2008). However, no data seem available on the production of similar enzymes by *Lecanicillium*.

Thus, degradative soil-inhabiting ascomycetes that produce extracellular ligninolytic enzymes can greatly contribute to the degradation of hazardous pollutants in the environment. Searching for new species and strains of ascomycetes with degradative properties and studying their physiology, biochemistry, and ecology may promote the understanding of the role these fungi play in the self-regeneration of natural ecosystems and may promote their use in biotechnology.

The aims of this work were as follows: (1) to examine the degradative activity of two newly isolated ascomycete strains, *Fusarium oxysporum* Schltdl. (IBPPM 543, MUT 4558) and *Lecanicillium aphanocladii* Zare & W. Gams (IBPPM 542, MUT 242), toward a range of hazardous pollutants; and (2) to detect ligninolytic enzymes in these two fungi.

Materials and methods

Organism isolation and identification

The fungi had been collected from the grounds of the Saratov oil refinery. The *Fusarium* strain had been isolated from creosote-contaminated railway sleeper wood, and the *Lecanicillium* strain had been isolated from a sporocarp of *Stropharia* sp., which had been collected from oiled litter. Samples were placed in petri dishes containing an agarized basidiomycetes rich medium (Bezalel et al. 1997) with our modifications (g/L): NH_4NO_3, 0.724; KH_2PO_4, 1.0; $MgSO_4 \times 7H_2O$, 1.0; KCl, 0.5; yeast extract, 0.5; $FeSO_4 \times 7H_2O$, 0.001; $ZnSO_4 \times 7H_2O$, 0.0028; $CaCl_2 \times 2H_2O$, 0.033; D-glucose, 10.0; peptone, 10.0. The dishes were maintained in the dark at 24–26°C for 4–6 days. The visually clear part of the mycelium was collected and placed in new petri dishes containing the same medium.

The strains were identified as *Fusarium oxysporum* Schltdl. (IBPPM 543, MUT 4558) and *Lecanicillium aphanocladii* Zare & W. Gams (IBPPM 542, MUT 242). A polyphasic approach was used that combined morpho-physiological observations of colonies with molecular tools. DNA was extracted with a NucleoSpin kit (Macherey Nagel GmbH, Duren, DE, USA) and was amplified by PCR with specific primers for the ITS1–5,8S–ITS2 region. PCR products were purified and sequenced at the Macrogen Europe Laboratory (Amsterdam, The Netherlands). The resulting sequences were compared with the reference sequences in the online databases provided by the CBS-KNAW Collection (Westerdijk Fungal Biodiversity Institute, The Netherlands) and by the National Center for Biotechnology Information (USA). Both fungi are maintained at the IBPPM RAS Collection of Rhizospheric Microorganisms (Saratov, Russia) and at the Mycotheca Universitatis Taurinensis (Turin, Italy). The accession numbers of the sequences deposited in GenBank are MG593980 for *F. oxysporum* Schltdl. (IBPPM 543, MUT 4558) and MG593981 for *L. aphanocladii* Zare & W. Gams (IBPPM 542, MUT 242).

Examination of degradative properties

The fungi were grown submerged at 24–26ºC in the basidiomycetes rich medium, or they were grown at the same temperature in Kirk's medium (Kirk et al. 1986) with our modifications (g/L): KH_2PO_4, 0.2; $MgSO_4 \times 7H_2O$, 0.05; $CaCl_2$, 0.01; thiamine, 0.0025; NH_4NO_3, 0.724. The pH of Kirk's medium was maintained with 25 mM phosphate buffer (pH 6.0), and 1% maltose was the carbon and energy source. Ligninolytic enzyme production was increased by supplementing Kirk's medium with 0.1% Tween 80 (Jager et al. 1985). The fungi were grown in 250-mL Erlenmeyer flasks, each containing 100 mL of either basidiomycetes rich medium or Kirk's medium. Growth was in the dark at 24–26 °C for 14 days with orbital shaking (120 rpm).

Pollutants were added on day 2 of fungal growth to final concentrations of 0.05 mg/L [PAHs (anthracene, phenanthrene, fluorene, pyrene, or fluoranthene) and anthraquinone dyes (Acid Blue 62 and Reactive Blue)], and 5 g/L [oil containing alkanes (47.4%), naphthene (22.3%), low-molecular-weight aromatic hydrocarbons (4.4%), high-molecular-weight aromatic hydrocarbons (5.4%), tars (3.9%), and pyrobitumen (16.6%)]. Anthracene, fluorene, phenanthrene, pyrene, fluoranthene, Acid Blue 62, and Reactive Blue were from Fluka (Switzerland); all the other compounds were from Reachim Co. (Russia). The PAHs were added as a chloroform stock solution (5 mg per 100 µl of chloroform). The control treatments contained 100 µl of chloroform, which too was added on day 2 of fungal growth. At intervals, the mycelium was separated from the growth medium by filtration. The increase in mycelium production (mg of dry biomass) was found by weighing. Residual PAHs and oil were extracted from the bulk of the flasks (without separation of the growth medium from mycelia) with chloroform (5 mL, three times). The resulting extracts were evaporated and were analyzed as described below.

Pollutant measurements

PAHs and their metabolites were analyzed by gas chromatography with a Shimadzu 2010 instrument equipped with a flame photometric detector. Compounds were separated with an HP5 column (Agilent), and helium was the carrier gas. The column temperature was kept at 200ºC for 3 min, then programmed to increase to 270ºC at a rate of 15ºC min^{-1}, and finally kept at 270ºC for 2 min. PAH oxidation products were identified with commercial 9,10-anthraquinone, phenanthrene-9,10-quinone, and 9-fluorenone (retention times, 4.51, 5.04, and 4.33 min, respectively) as the marker compounds. The gas chromatography analysis was carried out at the Simbioz Center for the Collective Use of Research Equipment in the Field of Physical–Chemical Biology and Nanobiotechnology (Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, Saratov).

Oil disappearance was measured by adsorption chromatography with gravimetric termination. Oil was extracted three times with 5 mL of chloroform, and the extracts were evaporated and applied to a column of Al_2O_3 (10 mL). After the oil was eluted with chloroform and dried to the complete evaporation of the solvent, the amount of oil was found gravimetrically (Polunina and Kushik 1977).

Because the basidiomycetes rich medium is optically opaque, the decolorization of dyes was studied on Kirk's medium at pH 6.0. Aliquots (2 mL) were taken from the flasks at intervals, and dye disappearance was monitored spectrophotometrically by the change in the absorption spectra at 590 nm (Pozdnyakova et al. 2015).

Enzyme activity measurements

Laccase activity was measured by the oxidation rate of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt (ABTS) at 436 nm (ε=29300 $M^{-1}cm^{-1}$; Niku-Paavola et al. 1988) and by the oxidation rate of syringaldazine at 525 nm (ε=65000 $M^{-1}cm^{-1}$; Leonowicz and Grzywnowicz 1981). Mn-peroxidase activity was measured by the oxidation rate of 2,6-dimethoxyphenol (DMP) with H_2O_2 and Mn^{2+} at 468 nm (ε=14800 $M^{-1}cm^{-1}$; Heinfling et al. 1998) and by the oxidation rate of 2,7-diaminofluorene (DAF) at 600 nm (ε=51000 $M^{-1}cm^{-1}$; Criquet et al. 2000) under the same conditions. Peroxidase activity was calculated as the difference between the values for DMP (or DAF) oxidation with and without H_2O_2. Lignin peroxidase was measured by the formation of the oxidation product of veratryl alcohol at 310 nm (ε=9300 $M^{-1}cm^{-1}$; Tien and Kirk 1984). One unit of enzyme activity (U/mL) is defined as the amount of enzyme that oxidizes 1 μM of substrate per min.

Emulsifying activity measurements

The emulsifying activity of the culture medium was measured according to Cooper and Goldenberg (1987). After fungal growth (with and without the pollutants) was completed, the growth medium was mixed 2:3 with kerosene, shaken for 20 min, and left to stand at room temperature for 48 h. The emulsifying activity (E_{48}) was calculated as the ratio of emulsion volume to total liquid volume and expressed as a percentage.

Statistics

All experiments were run in triplicate, each having been repeated at least three times. Standard statistical methods were used to calculate the mean values and standard deviations (SD). Data were processed with Microsoft Excel 2003.

Results and Discussion

Ascomycetes have a strong degradative potential toward natural compounds and pollutants. *L. aphanocladii* IBPPM 542 and *F. oxysporum* IBPPM 543 were isolated from the same source (creosote-contaminated sleepers) in a search for new fungal strains with degradative properties. The isolated strains were checked for their ability to degrade PAHs from creosote used to preserve railway sleepers. PAHs are priority environmental pollutants, and their degradation and transformation are well known (Kadri et al. 2017).

In our experiments, *F. oxysporum* had weak degradative activity toward PAHs. The least recalcitrant was the three-ring fluorene, with a removal value of 42% (Fig. 1A). Possibly, the low ionization potential (7.88 eV) and the relatively high solubility (1.98 mg/L) of fluorene make it accessible for fungal degradation. A metabolite from fluorene was found whose retention time (4.33 min) corresponded to that of commercial 9-fluorenone. Phenanthrene and fluoranthene were degraded by 21% and 26%, respectively. Finally, anthracene and pyrene were not degraded by this fungus.

Data are different on the degradation of PAHs by *Fusarium* strains. The ability to degrade anthracene is species specific: for example, *F. moniliforme* degraded about 77% of anthracene from 100 mg/L, while *F. solani* did not degrade anthracene at all (Krivobok et al. 1998). In some cases anthracene cannot be a single source of carbon and energy for *Fusarium* fungi, and the need for cometabolism was often reported.

The other fungus tested was *L. aphanocladii*. The reason for our interest in it is related to its typical ecological niche: *L. aphanocladii* is usually reported to be an entomopathogenic fungus. In this work, *L. aphanocladii* was isolated only with the traditional basidiomycetes rich medium containing peptone and yeast extract; therefore, it can be considered a saprotroph.

L. aphanocladii IBPPM 542 intensely degraded all the PAHs, removing 40 to 80% for three-ring PAHs (anthracene, phenanthrene, and fluorene) and about 50% of those with four condensed rings (pyrene, fluoranthene) (Fig. 1A). Some degradation metabolites were identified. These included 9,10-anthraquinone (retention time, 4.51 min), 9,10-phenanthrenequinone (retention time, 5.04 min), and 9-fluorenone (retention time, 4.33 min), the products of degradation of anthracene, phenanthrene, and fluorene, respectively.

The appearance of the degradation products and the disappearance of the substrate were checked only after 14 days of growth. In separate experiments, in which the growth time of both fungi was extended to 28 days, the degradation products disappeared and phthalic acid was simultaneously formed (data not presented).

Oil and oil products are ubiquitous and the most abundant environmental pollutants. Undoubtedly, ascomycetes, which are a large part of the soil microbiota, participate in the degradation and detoxification of

194 oil hydrocarbons. Both ascomycetes used in this study were active oil degraders, with 60% of oil transformed
195 with *L. aphanocladii* and 84% with *F. oxysporum* (Fig. 1A).

196 The degradation by both fungi of all pollutants used in this study was accompanied by the production
197 of an emulsifying compound(s). Emulsifying activity of the medium (E_{48}) varied from 6.2 to 41%, depending
198 on the fungal species and on pollutant solubility (Fig. 1B). No emulsifying activity was detected in the control
199 (pollutant-free) treatments. The production of a biosurfactant that increases the solubility of hydrophobic
200 compounds was found earlier in the basidiomycetes *Coriolus versicolor* and *Pleurotus ostreatus* (Arun et al.
201 2008; Nikiforova et al. 2009). Biosurfactant production by ascomycetes was described, too (Bhardwaj et al.
202 2013). However, this report is the first to describe the production of an emulsifying compound(s) by *Fusarium*
203 and *Lecanicillium* in response to the presence of hydrophobic compounds in their growth medium.

204 As said above, 9,10-anthraquinone was a metabolite from PAH degradation by the fungi tested. The
205 three-ring structure of anthraquinone occupies the centers of the molecules of synthetic anthraquinone-type dyes,
206 which are highly resistant to biodegradation (Eichlerova et al. 2007). Synthetic dyes are also hazardous
207 pollutants, entering the environment through runoff from paint-and-varnish and textile facilities.

208 Of the wide range of anthraquinone dyes available, those used most often as models are Poly R-478 and
209 Remazol Brilliant Blue SN4R (Liu et al. 2004). Dyes of this group can be used to screen fungi for the ability to
210 produce extracellular ligninolytic peroxidases and laccases.

211 In our experiments, both fungi decolorized the two anthraquinone-type dyes used, Acid Blue 62 and
212 Reactive Blue 4. As expected, *L. aphanocladii*, an active degrader of aromatic compounds, intensely decolorized
213 the dyes (to a level as high as 70%; Fig. 2).

214 Data on the decolorization activity of *F. oxysporum* and *L. aphanocladii* may indirectly point to the
215 production of ligninolytic enzymes by these fungi. Therefore, we next measured the activities of the main
216 ligninolytic enzymes (lignin peroxidase, Mn-peroxidase, and laccase) during pollutant degradation and in the
217 control (pollutant-free) treatments.

218 In the controls, no ligninolytic enzyme activity was found. In polluted environments, both fungi
219 produced one type of ligninolytic enzyme. The enzyme was active in the presence of H_2O_2, and 0.5 mM Mn^{2+}
220 produced a 2- to 3-times increase in its activity. The enzyme did not oxidize veratryl alcohol and
221 syringaldazine, the test substrates for lignin peroxidase and laccase, respectively. From these data, the enzyme
222 was tentatively identified as an Mn-peroxidase.

223 Fig. 3A1 presents the time course of Mn-peroxidase production by *F. oxysporum* (A) and *L.*
224 *aphanocladii* (B) during PAH degradation. The most active inducers of *F. oxysporum* peroxidase were
225 phenanthrene, fluoranthene, and/or their degradation products. Although anthracene and pyrene were not
226 actively degraded by *Fusarium*, they too induced peroxidase activity (Fig. 3A1). Fig. 3A2 shows the data on
227 polyacrylamide gel electrophoresis (PAGE) under nondenaturing conditions. The enzyme was detected only
228 after the gels had been stained with *o*-dianizidine in the presence of H_2O_2. Throughout growth, *F. oxysporum*
229 produced only one isoform of Mn-peroxidase, regardless of the PAH used.
230 *L. aphanocladii*, too, produced Mn-peroxidase in response to the presence of pollutants in the medium
231 (Fig. 3B1). Best peroxidase induction was achieved with anthracene and/or its degradation products. Only one
232 enzyme was detected by nondenaturing PAGE after the gels had been stained with *o*-dianizidine and H_2O_2 (Fig.
233 3B2). Throughout growth, *L. aphanocladii* produced only one isoform of Mn-peroxidase, regardless of PAH
234 used.
235 For crude enzyme preparations, the fungi were grown until peroxidase production was maximal, after
236 which the mycelium was separated from the culture medium by filtration and the culture liquid was concentrated
237 by ultrafiltration on Amicon PM10 and was used as a source of crude peroxidases. Some catalytic properties of
238 these peroxidases have been investigated. Both enzymes oxidized ABTS, DAF, and DMP only in the presence of
239 H_2O_2. The reaction was largely stimulated by Mn^{2+} – by 3 and 2 times for the peroxidases of *F. oxysporum* and
240 *L. aphanocladii*, respectively. These properties make these enzymes similar to the Mn-peroxidases of
241 ligninolytic basidiomycetes (Wong 2009).
242 The Ascomycota have been mainly studied for laccase production, and several reviews have
243 summarized laccase production and use on an industrial scale for the processing of paper pulp and for the
244 discoloration of dyes. Ascomycete laccases have been comprehensively studied and some have been sequenced
245 (Fernaud et al. 2006; Dekker et al. 2007; Castilho et al. 2009). *F. solani* produced three ligninolytic enzymes in
246 the presence of different inducers (Obruca et al. 2012). Because H_2O_2 significantly increased the activities of
247 Mn-peroxidase, lignin peroxidase, and laccase, the authors suggested that these enzymes can be involved in
248 stress response to H_2O_2 (Obruca et al. 2012). The participation of *Fusarium* enzymes in the degradation of PAHs
249 is still a matter of discussion. For example, Wua et al. (2010b), using *F. solani* to treat mangrove deposits
250 contaminated by PAHs, showed that laccase is involved in the degradation of anthracene and
251 benzo[a]anthracene, whereas lignin- and Mn- peroxidase were not detected. The laccase was isolated, purified,
252 and characterized (Wua et al. 2010a).

In *F. oxysporum* cultures used to transform the aromatic components of the dry residue from an olive mill, Sampedro et al. (2007) detected the activities of Mn-peroxidase and Mn-independent peroxidase but did not detect laccase activity. No information on Mn-peroxidase production by *Lecanicillium* seems available.

Conclusions

Both fungi were degradative toward a range of hazardous pollutants, including PAHs, anthraquinone-type dyes, and oil. Both produced peroxidase (presumably Mn-peroxidase) and an emulsifying compound(s) in response to the presence of the pollutants in the growth medium. These properties may be necessary for the survival of the fungi in polluted environments and for the degradation of pollutants. The physiological peculiarities of *Fusarium* and *Lecanicillium*, including the functions of the peroxidases and an emulsifying compound(s), and their participation in the natural cleansing of contaminated environments will be the subject of further work.

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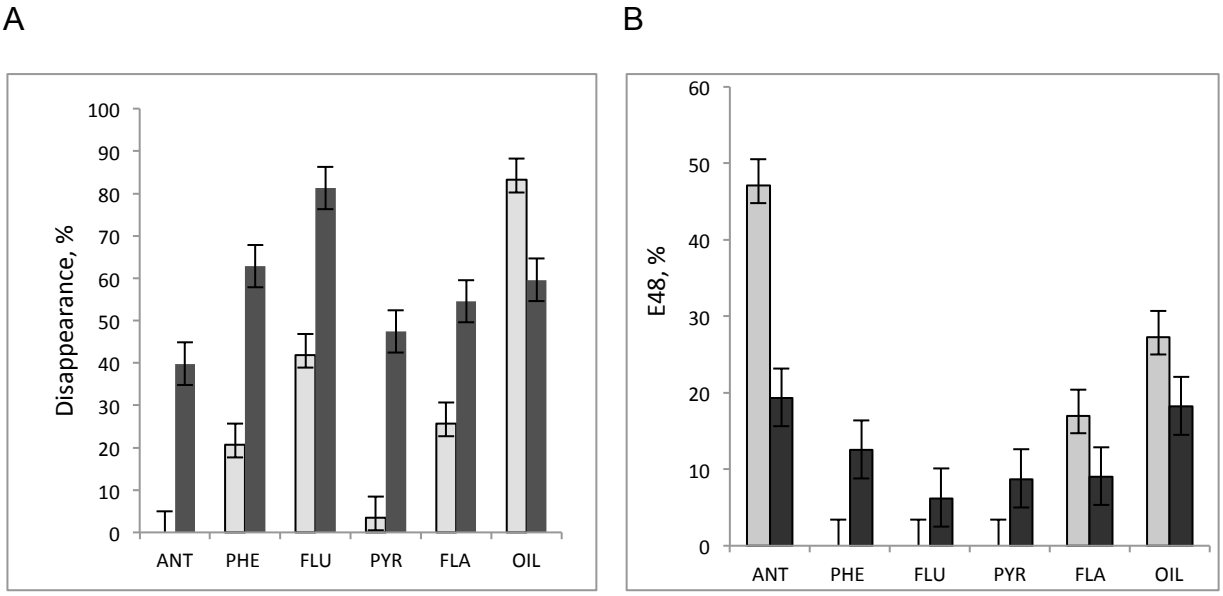
Table 1 Catalytic properties of *F. oxysporum* and *L. aphanocladii* peroxidases

Fungus	Activity, U/mL					
	$-Mn^{2+}$			$+Mn^{2+}$		
	ABTS	DMP	DAF	ABTS	DMP	DAF
F. oxysporum	2.8±0.7	4.1±0.5	3.6±0.4	9.5±1.6	14.0±2.4	12.2±0.8
L. aphanocladii	2.4±0.8	5.3±0.8	4.8±0.6	5.5±1.2	12.4±1.8	11.0±0.9



422 **Fig.1** Pollutant disappearance (A) and emulsifying activity production (B) during submerged cultivation of *F.*
423 *oxysporum* (□) and *L. aphanocladii* (■): ANT, anthracene; PHE, phenanthrene; FLU, fluorene; PYR, pyrene;
424 FLA, fluoranthene. Data always represent means ± standard deviations from triplicate cultures, $P \leq 0.05$.

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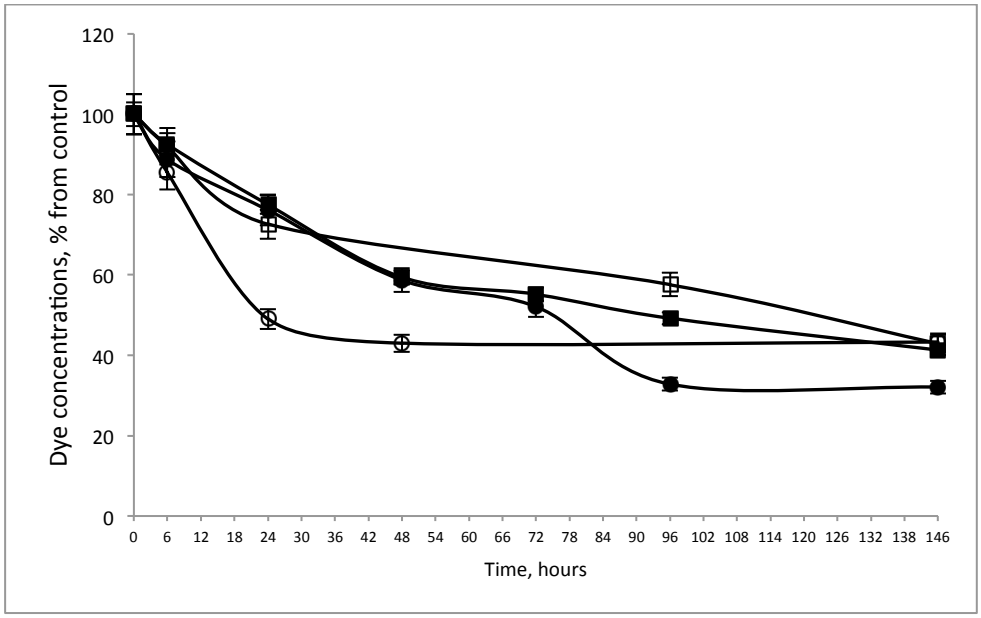

427 **Fig. 2** Decolorization of anthraquinone-type dyes by *F. oxysporum* (■, □) and *L. aphanocladii* (●○): Acid Blue
428 62 (■, ●) and Reactive Blue 4 (□, ○). Data always represent means ± standard deviations from triplicate cultures,
429 $P \leq 0.05$.

430

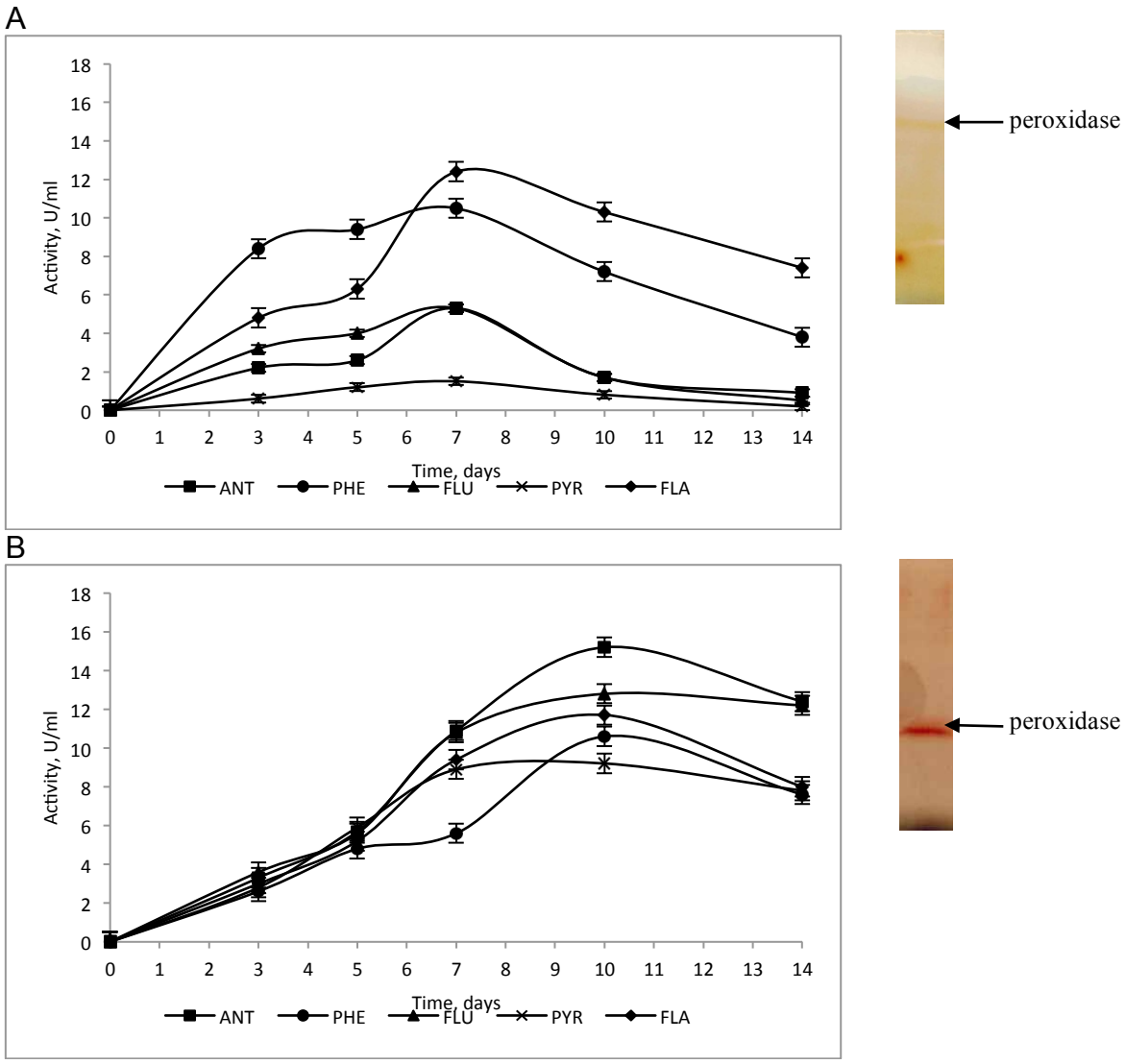

Fig. 3 Time course of Mn-peroxidase production by *F. oxysporum* (A1) and *L. aphanocladii* (B1) and nondenaturing PAGE of the detected enzymes (A2, B2). Data always represent means ± standard deviations from triplicate cultures, $P \leq 0.05$.