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DEGRADATIVE ACTIVITY AND PRODUCTION OF THE EXTRACELLULAR PEROXIDASES BY MICROMYCETES WITH DIFFERENT ECOLOGICAL STRATEGY

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Abstract

Environmental pollution by natural and man-made pollutants remains a serious problem. Agricultural areas are contaminated by major and hazardous pollutants such as oil, which comes from local oil-producing and oil-refining facilities, and polycyclic aromatic hydrocarbons (PAHs), which result from natural fires and from human activity associated with the use of flammable organic raw materials. This presents the hazard of accumulation of toxic substances in food and fodder plants. Natural ecosystems have powerful detoxifying potential, which is ensured by the degradative activity of microorganisms, including ascomycetes — one of the largest groups in the fungal kingdom. Here we examined the degradation of oil and PAHs by micromycetes with different ecological strategies and detected ligninolytic enzymes implicated in the oxidation of the pollutants. We used four ascomycete strains with different taxonomic affiliations and ecological strategies. These were *Fusarium oxysporum* IBPPM543, *Lecanicillium aphanocladii* IBPPM542, *Cladosporium herbarum* MUT3238, and *Geotrichum candidum* MUT4803. The fungi were grown in liquid media with different compositions that received additions of the pollutants used: oil, PAHs, and anthraquinone-type dyes. After 14 days of fungal growth, the elimination of the pollutants and the content of their main degradation products were examined by GC. Ligninolytic enzyme activity was estimated spectrophotometrically by the oxidation rate of the corresponding test substrates. All treatments in the experiments and analyses had no less than three replications, and each experiment was repeated no less than three times. Data were processed with Microsoft Excel 2003 software. All fungi oxidized oil; the utilization was from 46 to 82 % of the initial concentration of 5 g/l within 14 days. *C. herbarum* MUT 3238 metabolized all PAHs included in the study (anthracene, phenanthrene, and fluorene) almost completely (initial concentration, 0.05 g/l). *L. aphanocladii* IBPPM 542 degraded anthracene, phenanthrene, and fluorene by 40, 63, and 81 %, respectively. *F. oxysporum* IBPPM 543 utilized phenanthrene and fluorene only by 20 and 40 %, respectively. PAH degradation by *G. candidum* MUT4803 was not greater than 18 %. Anthracene was not degraded by *F. oxysporum* IBPPM 543 and *G. candidum* MUT4803. The degradation of the pollutants was accompanied by the production of extracellular peroxidases by all fungi except *G. candidum*. The activities of these peroxidases were largely stimulated by Mn^{2+}; this property makes them similar to the Mn-peroxidases of basidiomycetes. This is the first report on the production of extracellular peroxidases by *C. herbarum* and *L. aphanocladii*. Neither of the fungi produced lignin peroxidase or laccase. Identification of the PAH oxidation products allowed us to suggest a pathway for PAH degradation by the tested fungi with an extracellular Mn-peroxidase. The degradation proceeds through the formation of quinones and carboxylic acids (phthalic and 2,2′-diphenic), which indicates that the PAHs are utilized almost completely and that no toxic metabolites accumulate. The obtained results indicate that two widely distributed ascomycete species, *C. herbarum* and *F. oxysporum*, and a strain of the lesser-known and

poorly studied species *L. aphanocladii*, have degradative potential toward oil and PAHs, which presupposes their involvement in the self-cleaning of the environment from these pollutants. The detection of ligninolytic enzymes (Mn-peroxidases) and of the corresponding products of PAH degradation speaks in favor of an ecologically appropriate pathway for the utilization of PAHs, which reduces the negative consequences associated with the possible formation of toxic metabolites. In the *G. candidum* strain, the oxidation of oil and PAHs is possibly due to the activity of other enzymes, for example cytochrome P450 monooxygenase, because no ligninolytic enzymes have been found. In addition, it is highly possible that this strain has a "dye peroxidase", which requires a narrow range of substrates and catalyzes the degradation of anthraquinone dyes, as was also shown by us. The ability of all fungal strains to degrade pollutants makes them promising candidates for practical use in bioremediation and other biotechnologies.

Keywords: ascomycetes, *Fusarium oxysporum*, *Lecanicillium aphanocladii*, *Cladosporium herbarum*, biodegradation, polycyclic aromatic hydrocarbons, oil, ligninolytic enzymes, peroxidases

Environmental pollution by natural and man-made pollutants remains a serious problem. Agricultural areas are contaminated by major and hazardous pollutants such as oil and polycyclic aromatic hydrocarbons (PAHs): oil due to local oil-producing and oil-refining facilities [1], PAHs as a result of natural fires and human activity associated with the use of flammable organic raw materials [2]. This presents the hazard of the accumulation of toxic substances in fodder and food plants. Natural ecosystems have powerful detoxifying potential, which is ensured by the degradative activity of microorganisms, including ascomycetes, one of the largest groups in the fungal kingdom.

All species of the *Fusarium* genus are characterized by high metabolic activity and adaptive plasticity [3]. They are primarily known as harmful to agriculture, causing diseases and toxicoses in plants and animals. The *Fusarium oxysporum* species often serves as a model for studies of plant—pathogen interaction [4]. However, most of its representatives have a saprotrophic lifestyle in the soil, with using complex carbohydrates and lignocellulose as sources of nutrition. The strains that form a mutually beneficial symbiosis with plants and even protect them from diseases are known [5, 6]. The participation of *Fusarium* in the processes of pollutants degradation, including PAHs and oil [7, 8], is shown.

The fungi of *Cladosporium* genus make a significant contribution to the degradation of plant residues but can cause plant diseases, damage of hay and grain in storage, by releasing toxins hazardous for animals and humans. Not enough information is known about pollutants degradation with these fungi: *C. resinae* is described as creosote or kerosene fungus, corroding pumps; the ability to metabolize anthracene is shown in *C. herbarum* [9], fluoranthene is described for *C. sphaerospermum* [10].

Representatives of the *Geotrichum* and *Lecanicillium* genera inhabit other types of biotopes. *Geotrichum candidum* causes a variety of rots on organic loadings, including fruits, and is considered the opportunistic fungus, the causative agent of opportunistic mycoses. At the same time, strains of this species are used in the production of elite cheeses and a number of fermented milk products [11]. Data on the degradation properties of *Geotrichum* mainly concern the ability of this fungus to discolor synthetic dyes, including those containing condensed aromatic rings [12, 13], transform 2,4,6-trinitrotoluene [14] and detergents [15]. The *Lecanicillium aphanocladii* species (known as *Verticillium lecanii* until 2001) is described as entomopathogenic [16, 17] and parasitic upon other fungi [18]. Its degrading properties with regard to pollutants have not been studied virtually. The information about the representatives of the other species of this genus is known: *L. saksenae* is a pesticides destructor [19], *V. lecanii* is the destructor of 2,4-dichlorophenol, 2,4-dichlorophenoxyacetic acid [20], and anthracene [9].

The destruction of natural substances and xenobiotics by fungi is carried out with the help of extracellular and intracellular enzymes. The extracellular lig-

ninolytic enzymes, the laccases and peroxidases, are produced by many basidiomycetes and ascomycetes in the process of lignocellulose degradation and are often considered as key enzymes of pollutant degradation [21]. The reports about the production of similar enzymes in *L. aphanocladii* and *C. herbarum* were not found.

The intracellular enzymes involved in pollutant degradations are primarily represented by cytochrome P450-dependent monooxygenases (cytochrome P450-monooxygenases), which are present in the cells of fungi regardless of their ability to produce ligninolytic extracellular enzymes. It is assumed that the pathway of primary oxidation of PAHs by one or another enzyme depends on a number of conditions; in the case of the hydroxylation of the aromatic ring and a number of subsequent transformations catalyzed by cytochrome P450-monooxygenase, such powerful carcinogens as epoxides and transdihydrodiols can be generated. At the same time, oxidation of these substances mediated by peroxidase or laccase occurs with the formation of quinones, which are further metabolized by the fungus up to compounds that are less toxic than the original PAHs. Therefore, oxidation of PAHs by ligninolytic enzymes may be a more logical strategy for detoxifying the polluted environment [22].

Within this framework, it is interesting to evaluate not only the destructive activity of some ascomycetes, i.e. the constant and mass inhabitants of plant communities, characterizing their participation in the self-purification of the natural environment, but also to determine the presence of ligninolytic enzymes, lowering the environmental risks associated with the possible formation of toxic metabolites.

In this paper, the ability of a number of previously unexplored strains of ascomycetes of different generic assignment to destroy oil and PAHs actively, by producing extracellular peroxidases, was shown for the first time.

The work objective was to study the degradation of oil and polycyclic aromatic hydrocarbons by micromycetes with different environmental strategies, as well as to identify the ligninolytic enzymes involved in the oxidation of these pollutants.

Techniques. Four ascomycetes strains used were *Fusarium oxysporum* IBPPM543 (isolated from old creosoted wood tie; IBPPM, the Collection of rhizospheric microorganisms of the Institute of Biochemistry and Physiology of Plants and Microorganisms RAS), *Lecanicillium aphanocladii* IBPPM542 (isolated from sporocarp of basidiomycete *Lentinus* sp.), *Cladosporium herbarum* MUT3238 and *Geotrichum candidum* MUT4803 (obtained from the Mycotheca Universitatis Taurinensis, Turin, Italy).

Fungi were cultured in flasks in a rich medium for basidiomycetes containing (in g/l) NH_4NO_3 0.724, KH_2PO_4 1.0, $MgSO_4 \cdot 7H_2O$ 1.0, KCl 0.5, yeast extract 0.5, $FeSO_4 \cdot 7H_2O$ 0.01, $ZnSO_4 \cdot 7H_2O$ 0.0028, $CaCl_2 \cdot 2H_2O$ 0.033, glucose 10.0, peptone 10.0; pH 6.0 [23]. Due to the optical opacity of this medium, the Kirk [24] medium in the authors' modification was used to observe the discoloration of dyes containing (in g/l) KH_2PO_4 2.0, $MgSO_4$ 0.348, $CaCl_2 \cdot 2H_2O$ 0.143, NH_4NO_3 1.02; (in ml/l) microelement-containing solution 10, thiamine 0.5. The microelement-containing solution included (in g/l) nitrilotriacetate 1.5, $MgSO_4 \cdot 7H_2O$ 3.0, $MnSO_4 \cdot H_2O$ 0.5, NaCl 1.0, $FeSO_4 \cdot 7H_2O$ 0.1, $CoSO_4$ 0.1, $CaCl_2$ 0.082, $ZnSO_4$ 0.1, $CuSO_4 \cdot 5H_2O$ 0.01, $AlK(SO_4)_2$ 0.01, H_3BO_4 0.01, $NaMoO_4$ 0.01; 25 mM phosphate buffer for pH 6.0; maltose at a final concentration of 1% was the source of carbon and energy.

The degradation activity of fungi was evaluated with PAHs (anthracene, phenanthrene, and fluorene), anthraquinone synthetic dyes (Acid Blue 62 and Reactive Blue 4) and crude oil (alkane 47.4%, naphthenes 22.3%, low-molecular aromatic substances 4.4%, high-molecular aromatic substances 5.4%, resins

3.9%, asphaltenes 16.6%). PAHs and oil were introduced into the culture medium in the form of chloroform solution, anthraquinone dyes in the form of aqueous solution. The final concentration for PAHs and anthraquinone dyes was 0.05 g/l, for oil 5.0 g/l. The media were inoculated with 2-days fungi inoculate and cultured at 26 °C and aeration (120 rpm), after 2 days pollutants were introduced into the flasks, in the control variants 100 μl of solvent. After 14 days, a decrease in the amount of pollutants, the content of the main metabolic products and the activity of ligninolytic enzymes were estimated.

PAHs and their degradation products were extracted from the culture liquid by chloroform (three times by 5 ml), the extracts were combined, evaporated to dryness and analyzed by the gas-liquid chromatography (GLC) method on the GC-2010 chromatograph (Shimadzu Deutschland GmbH, Germany) with the flame photometric detector. The substances were separated on the HP5 column (Agilent Technologies Inc., USA), with carrier gas helium. The column temperature of 200 °C was maintained for 3 min and then increased up to 270 °C at the rate of 15 °C/min; this temperature was maintained for another 2 min. Prior to GLC, 2-carboxybenzaldehyde, 2,2′-diphenic and phthalic acid were methylated with CH_3COCl. Anthracene (retention time 4.15 min), 9,10-anthraquinone (5.39 min), phenanthrene (4.08 min), phenanthrene-9,10-quinone (6.86 min), fluorene (5.57 min), 9-fluorenone (4.33 min), 2-carboxybenzaldehyde (7.31 min), 2.2′-diphenic acid (6.7 min), phthalic acid (8.99 min) were used as markers for identification of PAHs and products of their oxidation.

The loss of dyes was tested spectrophotometrically, by taking 2 ml aliquots from the flasks at certain time intervals, followed by measurement of absorption at $\lambda = 590$ nm [25]. The residual oil from the culture medium was extracted with chloroform (three times by 5 ml); the extracts were combined and evaporated to dryness. The total oil content in the samples was determined by adsorption chromatography with gravimetric termination [26].

Enzyme activity was evaluated spectrophotometrically (Evolution 60, Thermo Scientific, USA): laccase by the oxidation rate of diammonium salt 2,2g-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) at $\lambda = 436$ nm [27]; Mn-peroxidase by the oxidation rate of 2,6-dimethoxyphenol in the presence of H_2O_2 and Mn^{2+} at $\lambda = 468$ nm [28]; lignin-peroxidase by the formation of the oxidation product of veratryl alcohol at $\lambda = 310$ nm [29]. Peroxidase activity was calculated as the difference between the rate of substrates oxidation in the presence of H_2O_2 and without it. The amount of enzyme that catalyzed the conversion of 1 μmol substrate per minute was taken as a unit of activity (U/ml).

Repetition in all variants in experiments and analyses was not less than 3-fold; each experiment was repeated at least 3 times. The obtained results were statistically processed in Microsoft Excel 2003. The figures show mean values (*M*) and standard deviations (±SD).

Results. The studied fungi had oil-oxidizing activity. In all variants, there was an intensive growth of biomass, the mycelium grew in the form of pellets of different sizes; after 14 days, the oil was completely emulsified. *C. herbarum* MUT 3238 and *F. oxysporum* IBPPM 543 destroyed oil by 82%, *L. aphanocladii* IBPPM 542 by 60%, *G. candidum* MUT4803 by 46%. The literature presents quite a lot of data on the participation of micromycetes in oil degradation [30-33]; however, no publications that would report on the oil-oxidizing properties of *L. aphanocladii* were found.

Concerning PAHs, the activity of fungi differed more brightly. For *C. herbarum*, the decrease in the initial amount of anthracene, phenanthrene, and fluorene from the culture medium for 14 days was almost complete.

L. aphanocladii degraded anthracene, phenanthrene, and fluorene by 40, 63, and 81% respectively. *F. oxysporum* oxidized phenanthrene and fluorene by only 20 and 40%, *G. candidum* destroyed no more than 18% of these PAHs. Anthracene was not degraded by the last two fungi (Fig. 1).

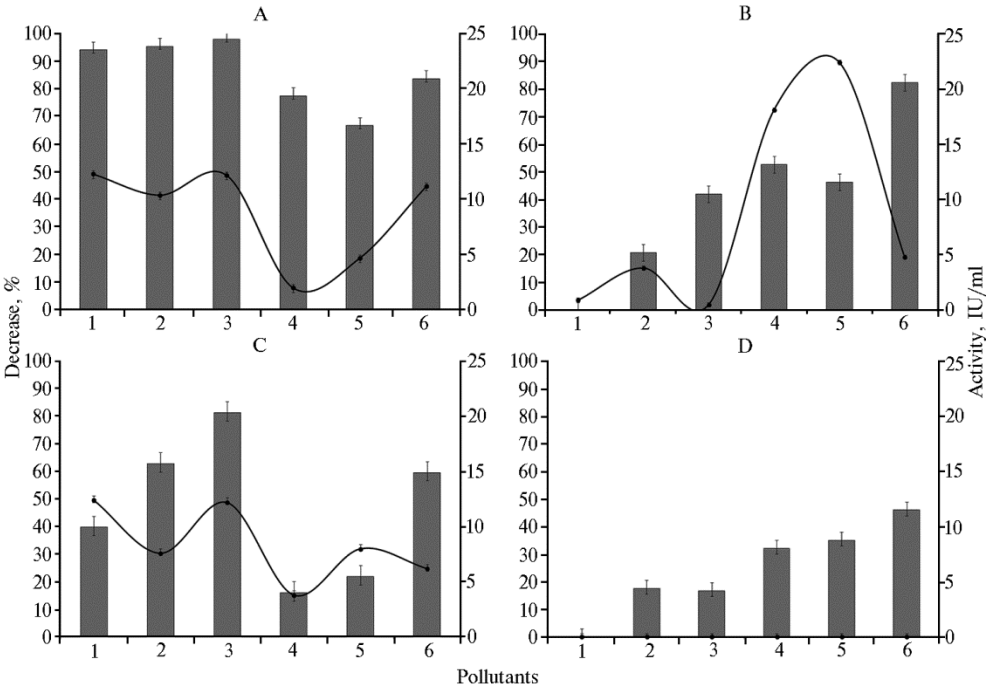

Fig. 1. Destructive activity (histogram) **for anthracene** (1)**, phenanthrene** (2)**, fluorene** (3)**, Reactive Blue 4** (4)**, Acid Blue 62** (5)**, oil** (6) **and extracellular peroxidase production** (graph) (day 14; $n = 3$, standard deviations).

A key role in the fungal degradation of various pollutants is attributed to extracellular oxidative enzymes, primarily ligninolytic [21]. In this regard, the studied strains were tested for the activity of laccase, lignin- and Mn-peroxidase. For primary screening of fungi for the production of these enzymes, their ability to discolor anthraquinone dyes is often used. As tests have shown, all four ascomycetes had this ability, which served as an indirect confirmation of the presence of such enzymes in them. However, in the conditions of this experiment, the activity of lignin-peroxidases and laccases in fungi was not detected. For three strains (except *G. candidum*), the presence of pollutants was accompanied by the production of extracellular peroxidases (see Fig. 1), which in control options (without pollutant) were absent. It was found that their activity was largely (up to 40%) stimulated by Mn^{2+} ions, which makes these enzymes similar to Mn-dependent peroxidases of basidiomycetes [34].

According to the literature, many micromycetes have ligninolytic enzymes. The representatives of the *Fusarium* genus produce Mn-dependent peroxidase, lignin-peroxidase and laccase involved in stress and degradation reactions of lignocellulose [35]. The role of laccases in the pathogenesis of fungi was confirmed [36]. The participation of the *Fusarium* enzymes in the degradation of PAHs was described in various options. For example, *F. solani* laccase is involved in the degradation of anthracene and benz(a)anthracene in mangroves polluted with PAHs, while lignin- and Mn-peroxidase were not detected [37]. When using *F. oxysporum* for the transformation of aromatic components in the dry waste of the olive mill, the activity of Mn-peroxidase and Mn-independent

peroxidase was detected, and the activity of laccase was not detected [38]. The members of the species *G. candidum* have three types of peroxidases, the participation of which in the process of degradation is widely discussed. These are lignin- and Mn-peroxidases [39, 40], as well as discoloring (dye-peroxidase) peroxidase, which is assumed to have a narrow substrate spectrum and serves as a key enzyme in the degradation of dyes, including those containing condensed aromatic rings [12, 13]. In this case, the revealed ability of the *G. candidum* strain to discolor anthraquinone dyes at very low oxidative activity against PAHs allows suggesting the presence of extracellular discoloration (dye)-peroxidase. In the publications of other authors, the information about the production of lignolytic enzymes by *L. aphanocladii* and *C. herbarum* was not found.

Certainly, in processes occurred in fungi, including the degradation of pollutants, the other enzyme systems, primarily oxygenases, which are known to be intracellular enzymes, are involved, and their participation is connected with the preliminary transportation of the substance into the cell. PAHs have low solubility, which probably determined the weak degradation of phenanthrene and fluorene (18%) by *G. candidum.*

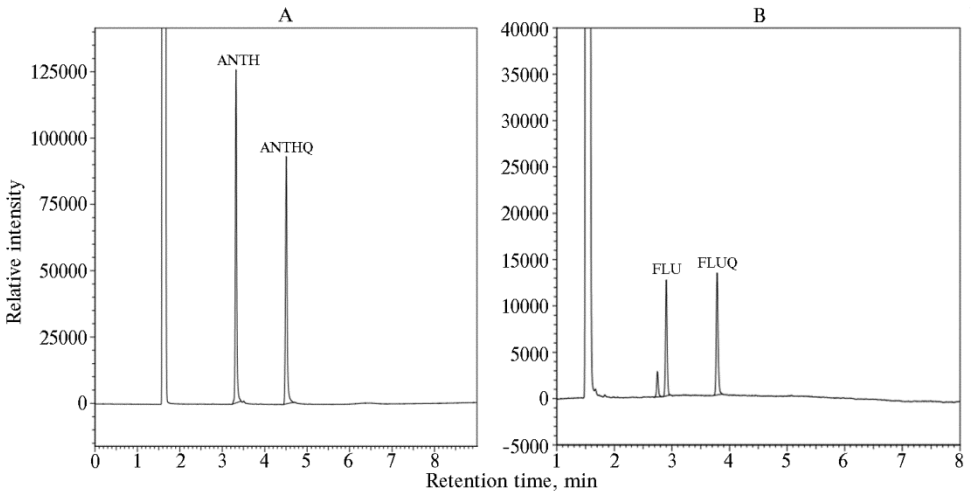

Fig. 2. Chromatograms of cultural liquid extracts of *Cladosporium herbarum* MUT 3238 after the degradation of anthracene (A) **and fluorene** (B)**:** the main peaks are ANTH — anthracene, ANTHQ — 9,10-anthraquinone, FLU — fluorene, FLUQ — 9-fluorenon (GC-2010 chromatograph, Shimadzu Deutschland GmbH, Germany; the flame photometric detector, column HP5, Agilent Technologies Inc., USA).

In the study of products of tricyclic PAHs anthracene and fluorene oxidation by the *C. herbarum* fungus with the GLC, the metabolite of anthracene degradation by *C. herbarum*, the 9,10-anthraquinone (Fig. 2), was identified, which after 14 days was almost completely destroyed with the formation of 2,2′-diphenic and phthalic acids. The metabolite of fluorene degradation by this fungus, the 9-fluorenol, was detected in trace amounts, which may be the result of rapid utilization of the initial PAH. As one of the final products of fluorene degradation, phthalic acid was found, which is known to be included in the main metabolism of fungi [41].

The formation and subsequent utilization of 9-fluorenol, 9-fluorenone and 2-carboxybenzaldehyde were observed in the degradation of fluorene by the *F. oxysporum*. Degradation of PAH by *C. herbarum* and *F. oxysporum* occurred with the formation and subsequent destruction of quinones, without the accumulation of toxic metabolites. It is necessary to note that the identified metabolites were identical to those found in basidiomycetes [42, 43]; this fact allows suggest-

ing the presence of similar metabolic pathways of PAH degradation for ascomycetes as well.

In the study of the PAH metabolism by *L. aphanocladii* and *G. candidum*, such unambiguous results were not obtained. In phenanthrene degradation by *L. aphanocladii*, trace amounts of phenanthrene-9,10-quinone were detected, which disappeared with an increase in the time of fungus culturing. The detection of this quinone and production of Mn-peroxidase by the strain allows us to suggest that *L. aphanocladii* has the pathway of PAH destruction which is similar to *C. herbarum* and *F. oxysporum*. Quinone metabolites during the degradation of PAHs by *G. candidum* were not identified.

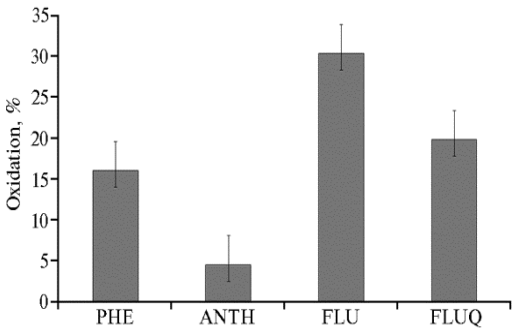

Fig. 3. Oxidation of polycyclic aromatic hydrocarbons by rough enzyme preparation of *Fusarium oxysporum* IBPPM 543 peroxidase: PHE — phenanthrene, ANTH — anthracene, FLU — fluorene, FLUQ — 9-fluorenone (day 2; $n = 3$, standard deviations are given).

As mentioned above, three of the four studied fungi produced peroxidase in response to the presence of pollutants in the culture medium. It is known that ligninolytic peroxidases are involved in the degradation of PAHs by oxidizing them to the corresponding quinones [21]. To clarify the role of the detected Mn-peroxidases of ascomycetes in PAH degradation, the direct oxidation reactions of three-cyclic PAH by an enzyme from *F. oxysporum* were investigated. A crude preparation of this enzyme was obtained, for which the fungal mycelium was cultured to a maximum of peroxidase production (20 U/ml); the culture medium was separated from the mycelium by filtration, concentrated 50-fold with ultrafiltration (Amicon PM-10 filter, Merck KGaA, Germany) and used as an enzyme source. It was found that this peroxidase oxidized both native PAH fluorene and phenanthrene, as well as 9-fluorenone, the oxidation product of fluorene (Fig. 3). Therefore, it is an extracellular fungal peroxidase that oxidized PAHs and also, at least, a fraction of polynuclear aromatic compounds of oil. It is important to note that in this experiment, fungi with different environmental strategies showed similar properties. All strains had high oil-oxidizing activity. Potentially entomopathogenic *L. aphanocladii* in terms of its destructive properties and production of Mn-peroxidase was similar to saprotrophs *C. herbarum* and *F. oxysporum*. The opportunistic *G. candidum* which did not show significant destructive activity against PAHs was significantly different from them, which may be the consequence of the absence of extracellular peroxidases similar to ligninolytic, although in the literature this species is referred to as a destructor of 2,4,6-trinitrotoluene [14]. *G. candidum* oxidizes oil and PAHs likely due to the activity of other enzymes, such as cytochrome P450-monooxygenase [44].

The obtained results give grounds to continue the research, using both classical biochemical methods of isolation, purification, and comprehensive study of enzymes and methods of pollutants degradation, and modern molecular biological approaches, making it possible to establish the presence and expression of the corresponding genes.

Thus, the representatives of two widely distributed in nature species of ascomycetes, *Cladosporium herbarum and Fusarium oxysporum*, as well as a strain of the less known and little studied species *Lecanicillium aphanocladii* have a high

destructive potential for oil and polycyclic aromatic hydrocarbons (PAHs), which implies the participation of these micromycetes in the processes of self-purification of natural ecosystems from pollutants. Identification of ligninolytic enzymes and related products of PAHs degradation indicates in favor of an environmentally appropriate way of PAHs utilization (with the formation of quinones), which reduces the negative consequences associated with the possible formation of toxic metabolites. The strain *Geotrichum candidum* has no ligninolytic enzymes and oxidation of oil and PAHs can be performed by other enzymes, such as cytochrome P450-monooxygenase. In addition, it is likely that this strain has a so-called discoloring peroxidase, which has a narrow spectrum of substrate specificity and catalyzes the discoloration of anthraquinone dyes. The ability of the studied strains to destroy pollutants makes them promising for practical use in bioremediation and other biotechnological processes.

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