II. ENVIRONMENTAL BIOTECHNOLOGY

BIODEGRADATION OF CRUDE OIL AND LIGNIN-MODIFYING ENZYME ACTIVITY OF WHITE ROT BASIDIOMYCETES

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Abstract. In the present study, the ability of eighteen white-rot basidiomycetes was evaluated for crude oil biodegradation. *Cerrena unicolor* strains, *Panus tigrinus* 433, *P. lecometei* 903, *Pleurotus ostreatus* 70, *Trametes maxima* 403, and *T. versicolor* 159 showed especially abundant mycelial growth on the surface of agar covered with droplets of crude oil. In the submerged cultivation in the glucose (3 g/L) containing medium, complete decolorization of indicator Resazurin was observed during two weeks in the presence of *Bjerkandera adusta* 139, *C. unicolor* 303, *Coriolopsis gallica* 142, *P. ostreatus* 70, *P. pulmonarius* 148, and *T. versicolor* 159. When artificially oil-polluted soil was inoculated with fungal mycelium pre-grown on a mixture of wheat straw and mandarin peels the maximum degradation rate (65%) was obtained when *C. unicolor* 305 was incubated in the 1% oil-containing soil for 28 days. At the same cultivation conditions, *P. ostreatus* 2175 eliminated 43.9% of initial oil when its concentration in the soil was increased to 2%. In the lignocellulose-containing soil, neither glucose nor yeast extract enhanced oil degradation, but wetting of soil with the distilled water to maintain its humidity favored oil elimination. The tested WRB secreted lignin-modifying enzymes in the presence of petroleum hydrocarbons; the higher was the concentration of lignocellulosic substrate in the soil the higher was the fungi enzyme activity. However, the data received did not show any direct relationship between the fungi enzyme activity and the degree of oil elimination.

Key words: mycoremediation, crude oil, basidiomycetes, cultivation conditions, lignin-modifying enzymes

INTRODUCTION

Intensive industrial and agricultural activity during the last century led to considerable contamination of soil and water with toxic organic pollutants having detrimental effects on the health of humans, animals, plants, and microbes. Soil, sediment, and groundwater contamination by petroleum and petrochemical derivatives due to their leaks and accidental spills during exploration, production, refining, transport, and storage is a widespread environmental problem [1-3]. Therefore, there is the need to develop low cost and efficient methods and strategies for their removal.

Bioremediation of the contaminated soils and effluents through exploitation of white rot basidiomycetes (WRB) potential is seen as a promising, environment friendly approach [4-6]. Compared to bacteria, these fungi possess required morphological and biochemical capacity to degrade a wide range of organic chemicals and use them as a growth substrate. Besides an ability of WRB to form extended mycelial networks and secrete hydrolytic enzymes to digest lignocellulosic growth substrates in their surroundings, these fungi possess the unique lignin-modifying enzymes (LME) including laccase, manganese peroxidase (MnP), versatile peroxidase (VP), and lignin peroxidase (LiP) having a broad substrate specificity [4-7]. Synergistic action of these and intracellular enzymes, in particular, cytochrome P450 monooxygenases make WRB well suited for degradation of high molecular-weight polycyclic aromatic hydrocarbons (PAH), total petroleum hydrocarbons (TPH), and bioremediation processes.

Recently, significant efforts were undertaken to screen and identify WRB strains that are most efficient in degrading TPH [5-8]. A large majority of the bioremediation investigations to date were done with *Phanerochaete chrysosporium* as the model microorganism [5, 9, 10]. This fungus showed the ability to remove more than 75–80% of TPH [5]. It was shown that polycyclic aromatic hydrocarbons with lower molecular weight, i.e. up to three rings, are fully degradable by *P. chrysosporium*, and heavier compounds with 5 rings or more resist degradation [10]. *Irpex lacteus*, *Phlebia radiata*, *Pleurotus ostreatus*, *Punctularia strigosozonata*, *Trametes versicolor*, and *Trichaptum biforme* were used for degradation of Bunker C fuel oil [6]. Moreover, addition of 10% of the spent mushroom compost colonized by *P. ostreatus* removed the petroleum hydrocarbons from contaminated soil in the presence or absence of soil enriching compounds over a short period of time [11]. However, there is a scarce information on the fungi cultivation conditions and factors affecting oil bioremediation process.

Recently, we have shown that many WRB isolated from various ecosystems of Georgia are capable to secrete high activities of LME in fermentation of plant raw materials [12, 13]. The objective of this study was to determine the ability of these fungi to degrade crude oil and utilize it as a carbon source for growth, to establish the cultivation conditions favoring these process, and to correlate fungal enzyme activity with oil removing potential.

doi.org/10.32006/eeep.2019.1.2636

MATERIALS AND METHODS

Organisms and inoculum preparation

The following WRB have been used in this study: *Cerrena unicolor* BCC300, BCC303, and BCC305, *Coriolopsis gallica* BCC142, *Fomes fomentarius* BCC38, *Funalia trogii* BCC146, *Lenzites betulina* BCC141, *Panus tigrinus* BCC433, *P. lecometei* BCC903, *Phanerochaete chrysosporium* ATCC24725, *Phlebia radiata* ATCC64658, *Pleurotus ostreatus* BCC70 and M2175, *P. pulmonarius* BCC148, *Pycnoporus sanguineus* BCC582, *Trametes maxima* BCC403, *T. multicolor* BCC511, and *T. versicolor* BCC159. All fungi are deposited in the Basidiomycetes Culture Collection of the Agricultural University of Georgia. Fungal inocula were prepared by growing mycelium on a rotary shaker at 150 rpm and 27°C in 250 mL flasks containing 100 mL of standard medium with the following composition (g/L): glucose - 15.0; peptone - 3.0; KH_2PO_4 - 1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ - 0.5; yeast extract - 3.0. The medium was adjusted to pH 6.0 with 2M NaOH. After seven days of the fungi cultivation mycelial pellets were harvested and homogenized with a Waring laboratory blender.

Collection and treatment of soil samples

Non-contaminated soil samples were randomly collected from the upper surface of soil (0-10 cm depth) on a territory of the Agricultural University of Georgia. The soil was air dried and then sieved through 2.5 mm mesh. The soil was sterilized through two successive autoclaving periods of 1h each on two consecutive days. The soil pH was determined by dissolving 1 g of soil in 5 mL of distilled water. The mixture was stirred well and allowed to stand for 30 minutes. Crude oil and Tween 80 were added to the soil in the final concentrations of 1.0-2.0% (w/w) and 0.1 % (v/w), respectively, and well mixed to make uniform contaminated soil. Crude oil was supplied from the Supsa oil terminal (Georgia). It was transferred to laboratory in dark glass bottle closed tightly and kept in a cold and dark place until to use.

Screening procedures

Initial screening experiments were performed using the agar medium containing (g/L): KH_2PO_4 - 1.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ - 0.5; NH_4NO_3 - 2.0; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ - 0.125, yeast extract - 3.0, agar - 15.0. Petri dishes were prepared by adding crude oil to warm agar medium. In order to have 1.5% concentration of petroleum hydrocarbon in all plates, the medium was thoroughly mixed with a magnetic stirrer, right before it was added to the plates. One

plug of eighteen selected WRB was inoculated on the surface of agar plates, sealed with Parafilm and incubated at 27°C. The same medium without inoculation was used as a control.

In parallel, the oil biodegradation potential of eighteen fungi was verified in their submerged cultivation using the following nutrient medium (g/L): crude oil - 10.0, KH_2PO_4 - 1.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ - 0.5; NH_4NO_3 - 2.0; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ - 0.125; Tween 80 - 1.0, redox-indicator Resazurin - 0.05, pH 6.0. This medium was supplemented with 3 g/L glucose (medium A) or 5 g/L yeast extract (medium B). Submerged cultivations were carried out in the rotary shakers Innova 44 (New Brunswick, USA) at 160 rpm and 27°C in 250 mL Erlenmeyer flasks filled with 50 mL of medium.

Crude oil degradation in soil

To evaluate the capability of selected fungi to eliminate crude oil hydrocarbons in an artificially polluted soil basidiomycetes were pre-grown in 100 mL flasks by the solid-state fermentation of a mixture of wheat straw (2 g) and mandarin peels (2 g) moistened with 10 mL of nutrient medium of following composition (g/L): KH_2PO_4 - 1.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ - 0.5; NH_4NO_3 - 2.0; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ - 0.250, pH 6.0. After sterilization, each flask was inoculated with 2 mL of homogenized mycelia and the cultivation of fungi was carried out in the dark at 27°C. After lignocellulosic substrate colonization (7-10 days), 2 g of the pre-cultured mycelial biomasses were inoculated into 100 mL flasks containing 20-30 g of preliminarily prepared oil-supplemented soil. Final concentration of crude oil in experiments was 1-2 g/100 g soil. The fungi cultivation was performed at 27°C in the dark for 2 and 4 weeks.

In an another set of experiments, 2 and 8 mL of homogenized mycelia were inoculated into 100 mL and 250 mL flasks containing, respectively, 3 g (1.5 g wheat straw + 1.5 g mandarin peels) and 15 g (7.5 g wheat straw + 7.5 g mandarin peels) moistened with 7.5 and 37.5 mL of nutrient medium of following composition (g/L): KH_2PO_4 - 5.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ - 2.5; peptone - 10.0; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (1.0 mM) - 1.25; yeast extract - 15.0, pH 6.0. The solid-state fermentation (SSF) of growth substrate was performed in the dark at 27°C until the full colonization of the substrate by mycelium (10 days). Crude oil and Tween 80 were added to the soil in the final concentrations of 1.5-2.0% (w/w) and 0.1 % (v/w), correspondingly, and mixed to make a uniformly contaminated soil. Then, 30 g of in this way prepared soil samples were added into 100 mL and 250 mL flasks containing fully

colonized by fungal mycelium substrate and the total content was well mixed to generate a lot of points of fungal growth. Flasks were incubated at 27°C in the dark for 2 and 4 weeks. Abiotic controls without fungal mycelia were prepared under the same condition.

All experiments were performed twice using three replicates at each time point. All results were expressed as the mean $\pm$ SD (standard deviation) with only $p \leq 0.05$ considered as statistically significant. The analyses were carried out using Microsoft Office Excel software.

Extraction and quantification of residual hydrocarbon

Usually, samples from control and experimental flasks were collected at time zero (after inoculation) and after 14 and 28 days of cultivation. 2 g of soil samples were mixed with 20 mL of dichloromethane, shaken on a rotary shaker during 30 minutes and allowed to stand for some time in order to separate the soil, then solvent phase was removed. This solvent extraction was repeated twice. All three extracts were pooled into the flask with anhydrous sodium sulfate to remove moisture. Then extracts were taken into the weighing bottle to evaporate dichloromethane in a hood. Two samples from each replicate were taken for crude oil extraction and further preparations. The gravimetric estimation of oil hydrocarbons remained after solvent evaporation was made by weighing the quantity of oil in a weighing bottle. The amount of the total petroleum hydrocarbons (TPH) degradation was calculated as follows:

% of oil degradation = $\frac{\text{initial concentration} - \text{final concentration}}{\text{initial concentration}} \times 100$

Extraction and assay of enzymatic activity

After the fungi cultivation, 2 g of each sample were extracted twice with 10 mL of distilled water.

Then extracts were filtered through nylon cloth and centrifuged at 6000 rpm during 15 minutes at 4°C. Supernatants were used for assay of enzyme activities and medium pH.

Laccase activity was determined spectrophotometrically at 420 nm as the rate of 0.25 mM ABTS (2,2'-azino-bis-(3-ethylthiazoline-6-sulfonate)) oxidation in 50 mM Na-acetate buffer (pH 3.8) at room temperature [14]. Manganese peroxidase (MnP) activity was measured at 270 nm by following the formation of a Mn^{3+} -malonate-complex [15] and by oxidation of Phenol Red [16] in the presence of 0.1 mM H_2O_2 . Lignin peroxidase (LiP) activity was determined spectrophotometrically at 310 nm by the rate of oxidation of 2 mM veratryl alcohol in 0.1 M sodium tartrate buffer (pH 3.0) with 0.2 mM hydrogen peroxide [17]. One unit (U) of LME activity was defined as the amount of enzyme that oxidized 1 μmol of substrate per minute.

RESULTS

Screening of petroleum hydrocarbons-degrading fungi

At the first stage, eighteen WRB strains belonging to different taxonomic groups were evaluated for their ability to grow on the oil contaminated agar medium and to eliminate the pollutant' hydrocarbons. Among the tested fungi, *C. unicolor* strains, *P. tigrinus* 433, *P. lecometei* 903, *P. ostreatus* 70, *T. maxima* 403, *T. versicolor* 159 showed especially abundant mycelial growth on the surface of agar covered with droplets of crude oil. It is obviously that petroleum did not inhibit the growth of these fungi; on the contrary, these WRB used oil compounds as nutrients. Concerning crude oil elimination, *C. gallica* 142 and *P. tigrinus* 433 (Fig. 1) as well as *P. lecometei* 903, *P. ostreatus* 70, *L. betulina* 141, *C. unicolor* 303 appeared to be most active in this process.

Fig. 1. Oil elimination in cultivation of 1) *C. gallica* 142 and 2) *P. tigrinus* 433 on agar plates

Fig. 2. The color change of Resazurin in the submerged cultivation of *C. gallica* BCC142 (2), *C. unicolor* BCC303 (3), *P. lecometei* BCC903 (10), and *P. chrysosporium* ATCC24725 (12) in the presence of crude oil and 0.3% glucose for 14 days

Subsequently, the screening of fungi for bioremediation of crude oil was continued in the submerged cultivation using redox indicator technique where Resazurin is an electron acceptor and acts as an indicator. During biodegradation of crude oil, it is irreversibly reduced to the pink color owing formation of acidic compounds from degraded oil and the color of indicator is changed from blue (oxidized) to colorless (reduced), what permits to ascertain the ability of the fungi to utilize the substrate. So, changes in color of inoculated media from deep blue

to colorless indicate the ability of fungi to biodegrade the crude oil.

Screening of basidiomycete strains under the submerged cultivation condition was performed in the mineral medium supplemented with 0.3% glucose (medium A) or 0.5% yeast extract (medium B). No changes in the medium pH and the indicator color were observed during 14 days of incubation of the abiotic control medium. When the fungi were cultivated during two weeks in the glucose-containing medium complete decolorization of indicator

Table 1. Basidiomycetes enzyme activity in the submerged cultivation in the presence of crude oil and redox-indicator

Fungi	Laccase (U/mL)		MnP ₂₇₀ (U/mL)		LiP (U/mL)		Indicator decolorization	
	Nutrient media							
	A	B	A	B	A	B	A	B
Control							-	-
<i>C. gallica</i> 142	7.5	0.1	0	0	0	0	++++	+++
<i>C. unicolor</i> 303	10.1	11.1	0.19	0.71	0	0	++++	++++
<i>F. fomentarius</i> 38	2.5	0.1	0	0	0	0	+++	+
<i>F. trogii</i> 146	0.9	0.3	0	0	0	0.03	++++	+
<i>L. betulina</i> 141	1.1	0.1	0	0	0	0.04	++++	+
<i>P. tigrinus</i> 433	0.1	0.3	0	0	0	0.04	++	++
<i>P. lecometei</i> 903	1.4	0.2	0.18	0	0	0	++	+
<i>P. chrysosporium</i> 24725	0	0	0	0	0	0	+	+
<i>P. ostreatus</i> 70	0.6	1.2	0	0	0	0	++++	++
<i>P. pulmonarius</i> 148	2.7	6.8	0	0.11	0	0	++++	++
<i>T. versicolor</i> 159	6.3	1.7	0.09	0	0	0	++++	+

was observed in the presence of following cultures: *B. adusta* 139, *C. unicolor* 303, *L. betulina* 141, *P. ostreatus* 70, *P. ostreatus* 2175, *P. pulmonarius* 148, and *T. versicolor* 159 (Fig.2). *P. tigrinus* 433 and *P. lecometi* 903 showed lesser efficiency in discoloration of Resazurin while *P. chrysosporium* 24725 practically failed in reducing the indicator color. It is worth noting that the tested strains of basidiomycetes decolorized Resazurin more efficiently in the glucose-containing medium than in the medium with yeast extract. In this case, only *C. unicolor* 303 was capable to completely decolorize indicator already after 7 days of cultivation.

It was necessary to evaluate the basidiomycetes lignin-modifying enzymes activities and correlate them with the Resazurin color changes. Among the fungi tested, *C. unicolor* 303 expressed the highest laccase activity during entire period of cultivation in both glucose or yeast extract supplemented media

(Table 1). Comparatively high enzyme activity was also detected in cultivation of *P. pulmonarius* 148 and *T. versicolor* 159 in the both media. It should be noted that *P. pulmonarius* 148 secreted significantly higher laccase activity in the yeast extract containing medium. However, *C. gallica* 142, *F. fomentarius* 38, *F. trogii* 146, *L. betulina* 141, and *P. lecometei* 903 showed higher laccase activity in the glucose containing medium expressing very poor enzyme activity in the absence of this carbon source. Only five fungal strains expressed comparatively low manganese-oxidizing MnP activity. The measurement of LiP activity revealed that only three fungi secreted traces of this enzyme activity in their cultivation in yeast extract containing medium without glucose. It is remarkable that no activity of tested enzymes was detected in the submerged cultivation of *P. chrysosporium* 24725.

Fig. 3. Elimination of crude oil in soil after 2 and 4 weeks' incubation with white-rot basidiomycetes (initial concentration of crude oil 204 mg/20 g soil)

Screening of petroleum hydrocarbons-degrading fungi in oil polluted soil

Subsequently, the capability of selected fungi to eliminate hydrocarbons in an artificially crude oil-polluted soil was studied. In the first set of experiments, seven strains of basidiomycetes pre-grown by SSF of a mixture of wheat straw and mandarin peels rapidly colonized soil during one

week. It is interesting that incubation of fungi during the first two weeks accompanied with an increase of soil pH from 5.4-5.5 to 5.8-6.5 followed by slight decrease by the end of experiment.

Determination of crude oil concentration after two-week incubation with fungi showed that all the tested WRB have a potential to eliminate this pollutant, but with a different degree. Among the selected fungi, *P. lecometei* 903 followed by *T.*

versicolor 159 and *P. sanguineus* 582 appeared to be the most potent oil destructors eliminating 94-98.1 mg of initial content (initial concentration was 204 mg/20 g soil) of pollutant. Respectively, these fungi eliminated 46-48% of initial oil content from the soil during 2 weeks' incubation (Fig. 3). However, further incubation of fungi in the oil-polluted soil

resulted in slowing of rate of the pollutant elimination by the majority of tested fungi. At the same time, *C. unicolor* 305 and *C. gallica* 142 appeared to be the poorest oil eliminators during the first stage of cultivation, but they were the most active destructors at the final stage of incubation removing 65 and 60% of the initial oil.

Table 2. LME activity in the cultivation of fungi in oil-contaminated soil

Fungi	Laccase (U/mL)			MnP ₆₁₀ (U/mL)		LiP (U/mL)
	Zero time	2 weeks	4 weeks	Zero time	2 weeks	Zero time
<i>C. unicolor</i> 300	1.49±0.40	0.03±0	0.03±0.01	0.05±0.01	0	0.07±0.01
<i>C. unicolor</i> 305	3.12±0.61	0.13±0.03	0.05±0.01	0.05±0.01	0.03±0.01	0.04±0.01
<i>C. gallica</i> 142	0.42±0.11	0.23±0.03	0.05±0.01	0.04±0.01	0.03±0	0.05±0.01
<i>P. lecometei</i> 903	0.46±0.09	0.08±0.02	0	Traces [†]	Traces	0
<i>P. radiata</i> 64658	0.10±0.03	0.03±0	0	0	0	Traces
<i>P. sanguineus</i> 582	0.38±0.12	0.03±0.01	0	0	0	0
<i>T. versicolor</i> 159	1.45±0.32	0.12±0.02	0.07±0.01	0.06±0.02	0	0

[†]Activities lower than 0.02 U/mL

Examination of the fungi enzyme activity during incubation in the oil-polluted soil showed their common features and diversity. Firstly, pre-grown on lignocellulosic substrate inoculum provided the tested cultures with the greatest enzyme activity from the beginning of cultivation. However, these cultures received qualitatively and quantitatively unequal enzyme activities. In particular, *C. unicolor* and *C. gallica* 142 possessed activity of three enzymes with the highest laccase activity in *C. unicolor* 305, *P. lecometei* 903 and *T. versicolor* 159

possessed both laccase and MnP activities, the model ligninolytic fungus *P. radiata* ATCC 64658 had low laccase and traces of LiP activities, whereas *P. sanguineus* 582 contained only comparatively low laccase activity (Table 2). Secondly, all the fungi showed manifold decreased enzyme activity already after two weeks of cultivation and complete disappearance of LiP activity. Moreover, after four weeks of incubation, with the exclusion of laccase, no activity of other enzymes was detected in the tested cultures.

Table 3. Degradation of crude oil in polluted soil and *Cerrena unicolor* 305, *Pycnoporus sanguineus* 582 and *Pleurotus ostreatus* 2175 enzyme activities

Samples	Degraded oil, %		Laccase (U/mL)		
	After 2 weeks	After 4 weeks	At time of inoculation	After 2 weeks	After 4 weeks
Abiotic control (3 g substrate)	3.4±0.3	5.9±0.7			
Abiotic control (15 g substrate)	5.5±0.6	9.0±1.1			
<i>C. unicolor</i> 305					
30 g soil + 3 g substrate	21.4±1.4	29.6±3.2	0.92±0.15	0.34±0.04	0.38±0.06
30 g soil + 15 g substrate	16.9±1.6	28.1±2.5	3.21±0.57	4.87±0.92	1.78±0.30
<i>P. sanguineus</i> 582					
30 g soil + 3 g substrate	22.1±1.5	31.8±2.7	0.09±0.02	0.15±0.02	0.09±0.02
30 g soil + 15 g substrate	16.8±1.8	28.5±3.0	0.13±0.03	0.25±0.05	0.18±0.03
<i>P. ostreatus</i> 2175					
30 g soil + 3 g substrate	30.2±2.4	43.9±3.1	0.10±0.01	0.07±0.01	0.07±0.01
30 g soil + 15 g substrate	22.6±2.0	40.9±3.5	0.16±0.02	0.11±0.02	0.13±0.02

To establish the role of growth substrate (i. e. nutrients) rate and sufficiency, the cultivation of selected fungi was carried out in 30 g soil mixed with 2% crude oil and 3 and 15 g of fermented substrate. In this set of experiments, evaluation of efficiency of crude oil hydrocarbons degradation in polluted soil was performed exploiting three basidiomycete strains with different enzymatic systems: *C. unicolor* 303 (the best producer of laccase, MnP and LiP), *P. sanguineus* 582 (produces only laccase) and *P. ostreatus* 2175 (commercial strain of oyster mushroom producing laccase, MnP and versatile peroxidase). The selected fungi showed an abundant biomass accumulation in the presence of crude oil and the media pH was appropriate for their growth.

Data in Table 3 show that natural process of oil degradation took place - after 2 and 4 weeks' incubation 3.4% and 5.9% of initial oil was lost by 3 g abiotic control while in the 15 g substrate the oil content decreased by 5.5% and 9%, respectively. Nevertheless, biotic variants showed significant

increase in the elimination of crude oil. Among the three fungi tested, *P. ostreatus* 2175 appeared to be especially efficient in pollutant degradation decreasing the oil content by 30.2-22.6% after 14 days' incubation and by 40.9-43.9% after 4 weeks' incubation.

Measurement of the fungi lignin-modifying enzyme activity showed that all fungi secreted laccase activity during the entire period of incubation (Table 3). As expected, only *C. unicolor* expressed low LiP activity after two weeks of incubation and phenol red-oxidizing MnP activity during the entire period of cultivation (data not shown) while other fungi did not show a capability to synthesis this enzyme. This results may be due to medium composition and absence of compounds stimulating enzyme production or by strong, irreversible sorption of enzymes on soil and substrate. However, it is not excluded that secretion of these enzymes took place during intermediate period between sampling time.

Table 4. Biodegradation of crude oil and *C. unicolor* 305 enzyme activity in SSF of lignocellulosic substrates

Condition of experiment	Days	Degraded oil, %	Laccase (U/mL)	MnP ₆₁₀ (U/mL)	LiP (U/mL)
Abiotic control	0	0	-	-	-
	14	3.9±0.2	-	-	-
	28	7.1±0.5	-	-	-
Biotic control (BC)	0	0.8±0.1	1.07±0.15	0	0
	14	20.5±0.3	0.62±0.07	0.07±0.01	0
	28	27.7±0.4	0.71±0.09	0.05±0.01	0.05±0.01
BC + 2 mL H ₂ O after 14 days	28	32.4±0.5	1.20±0.19	0.04±0.01	0
BC + 1 g glucose after 14 days	28	30.4±0.6	1.23±0.20	0.05±0.01	0
BC + 0.5 g yeast extract after 14 days	28	28.1±0.4	0.49±0.07	Traces [†]	0
BC + 0.25 g CuSO ₄ after 14 days	28	29.8±0.5	3.30±0.45	0	0
BC + mixture of all additives after 14 days	28	28.2±0.6	5.13±0.73	Traces	0

[†]Activities lower than 0.02 U/mL

The previous experiments revealed that the crude oil biodegradation occurred comparatively actively during the first two weeks of SSF, then the process slowed down. We assumed that the main reasons of this situation are the substrate' humidity decrease and a limitation of nutrients required for the fungal growth. Therefore, biodegradation of petroleum hydrocarbons by *C. unicolor* 305 actively producing LME was studied in the same conditions of SSF of lignocellulosic substrates using some additives to support the fungus growth and to stimulate process of oil degradation after two weeks of cultivation. In this experiments, a mixture of

wheat straw (5 g) and mandarin peels (5 g) moistened with 25 mL of nutrient medium was used.

After two weeks of the fungus cultivation, the media were supplemented with 2 mL of distilled water or 2 mL of solutions containing 1 g glucose (as an additional carbon source), 0.5 g yeast extract (as a source of additional nitrogen and vitamins), 0.25 g CuSO₄·5H₂O (1 mM, to induce laccase synthesis) or mixture of all additives. The content of each flask was mixed and flasks were incubated under above-mentioned conditions for two more weeks. Results in Table 4 show that the abiotic control lost 3.9% and 7.1% of the initial oil after 14

and 28 days of incubation, respectively. In the biotic variant, the SSF of lignocellulosic substrates by *C. unicolor* 305 accompanied with 20.5% loss of initial oil after two weeks and 27.7% after 4 weeks of SSF (only 7.2% during the last two weeks). Additional wetting of the substrate after 14 days of SSF increased the quantity of degraded oil to 32.4%, i.e. by 11.9% during two last weeks of incubation. However, neither additional carbon nor nitrogen source (or their mixture) did not further stimulate the pollutant elimination. It is interesting that addition of water or glucose solution almost two-fold increased the fungus laccase activity as compared with the biotic control while supplementation of the yeast extract rather decreased it. As expected, addition of copper favored laccase production 5-fold increasing enzyme activity as compared with the control culture. Moreover, the presence of all additives simultaneously more than 8-fold stimulated laccase production but without any positive effect on the crude oil degradation. On the contrary, the data received evidence that the medium supplementation with the individual additives or with their mixture did not favor the peroxidases production.

DISCUSSION

In this study, recently discovered and comprehensively characterized for LME production WRB strains belonging to various taxonomic groups and having different sets of individual enzymes have been for the first time screened for their potential to eliminate oil hydrocarbons. For the initial screening the agar plate method was exploited which has been extensively used in other studies on isolating the hydrocarbons-degrading fungi [18, 19]. Screening of eighteen WRB strains revealed several fungi actively degrading crude oil. Among these fungi, *C. unicolor* 303, *C. gallica* 142, *L. betulina* 141, *P. tigrinus* 433, *P. lecometei* 903, *P. ostreatus* 70 showed especially abundant mycelial growth on the surface of agar covered with droplets of crude oil and appeared to be the most active in oil removal using it as a nutrient. Screening of WRB for bioremediation of crude oil in the submerged cultivation in the low glucose-containing medium using the redox indicator Resazurin confirmed the bioremediation potential of the above-mentioned fungi and in addition revealed *F. trogii* 146, *P. pulmonarius* 148, and *T. versicolor* 159 abilities to degrade oil. Unexpectedly, the model strain, *P. chrysosporium* 24725, practically failed in reducing the indicator color. It is possible that the presence of crude oil or Resazurin inhibited enzyme production

by this fungus. However, we assume that the medium composition and cultivation conditions did not favor LME production by *P. chrysosporium* 24725 since no enzyme activity was detected in its submerged cultivation. By contrast, *C. unicolor* 303 followed by *C. gallica* 142, *P. pulmonarius* 148 and *T. versicolor* 159 expressed high laccase activity during entire period of cultivation. It is worth noting that all fungi secreting high laccase activity successfully degraded oil but some fungi (*F. trogii* 146, *L. betulina* 141, and *P. ostreatus* 70) accumulating comparatively low laccase activity, nevertheless, actively removed the pollutant. It is possible that even low laccase activity is sufficient to provide the oil degradation by growing cultures. However, it is not ruled out that the time of enzyme activity measurement in these experiments (7 and 14 days) did not match the fungi maximum activity.

When elimination of oil hydrocarbons in an artificially oil-polluted soil was studied using inoculum pre-grown by SSF of lignocellulose, *P. lecometei* 903 followed by *T. versicolor* 159 and *P. sanguineus* 582 appeared to be the most potent oil destructors eliminating 46-48% of initial oil content from the soil during 2 weeks' cultivation. Usually, further incubation of fungi in the oil-polluted soil resulted in slowing of rate of the pollutant elimination by the majority of tested fungi. This phenomenon can be explained by several reason. Firstly, the fungal growth slowed because exhaustion of main nutrients and they became metabolically less active. Secondly, it is possible that during an initial growth the fungi primarily utilized easily metabolizable oil fractions while during the second stage of incubation they degraded more recalcitrant oil fractions. Thirdly, it is possible that environmental conditions (medium pH, humidity) became unfavorable for fungal growth and pollutant elimination. In particular, measurement of the fungi enzyme activity revealed manifold lower laccase and MnP activities in two-weeks cultures as compared with enzyme activity at the time of inoculation (Table 2). Moreover, no LiP activity was detected after 14 days of fungi cultivation.

To test these hypothesis, two sets of experiments were performed. In particular, the rate of inoculum (and growth substrate) of three WRB pre-grown on lignocellulose was 3 g and 15 g per 30 g soil. Among the fungi tested, *P. ostreatus* 2175 appeared to be especially efficient in pollutant degradation decreasing the oil content by 40.9-43.9% after 4 weeks' incubation. Taking into account the literature

data on the crude oil degradation performances and the competitiveness of oyster mushroom in soil [20, 21], our finding that *P. ostreatus* 2175 appeared to be a promising eliminator of crude oil is encouraging since this strain is widely exploiting for industrial production of oyster mushroom and its spent substrate can be successfully used in bioremediation of polluted soils.

The data received in this study evidence that independently on the inoculum and growth substrate rate, the crude oil degradation was the most intensive during the first two weeks of fungi cultivation (Table 3). As shown, an enrichment of the polluted soil with the fermented growth substrate by increasing of the inoculum rate from 10% to 50% delayed the oil degradation during this period obviously due to the presence of more easily consumable and preferable nutrients. However, the quantity of crude oil eliminated by fungi during the subsequent two weeks of incubation in the enriched medium exceeded that in the medium containing 30 g soil + 3 g substrate. Thus, enrichment of 30 g oil-polluted soil with 15 g mycelial biomass instead 3 g increased the quantity of oil eliminated by *C. unicolor* 303, *P. sanguineus* 582, and *P. ostreatus* 2175 during the last two weeks of cultivation from 8.2, 9.7, and 13.7% to 11.2, 11.7, and 18.3%, respectively. Analogically, it was demonstrated that the agro-wastes in single and combined forms possessed the nutritional components required by the microorganisms to attack and break-down different hydrocarbon components in the soils [22]. Moreover, the reduction in the total petroleum hydrocarbons (THC) of the soil by indigenous microorganisms was dose dependent: the higher was the treatment levels the lower was the THC content in the soils. Alotaibi et al. [23] also showed that wheat straw when applied to crude oil-contaminated soil enhanced the biodegradability of pollutant, especially at contamination levels of up to 100-150 mL crude oil/kg. Consequently, we can conclude that supplementation of soil with lignocellulosic material at a certain rate extends the bioremediation process carried out by the fungi. This conclusion is in an agreement with several studies indicating that degradation of petrochemical compounds requires the presence of another carbon source [20]. This might be due to co-metabolism processes that play a role in significant removal of petroleum hydrocarbon, specifically for higher fractions [24]. Nevertheless, unlike *Daedaleopsis* sp. [8], supplementation of medium with an easily metabolizable carbon source (glucose) or nitrogen

source (yeast extract) in this work did not stimulate the pollutant elimination. Nevertheless, the addition of glucose as well as copper to the growing culture significantly promoted the fungus growth and enzyme production; one can assume that maintaining of a fungal culture enzymatic activity during incubation will favor the bioremediation process. It is worth noting that among several supplements tested in this study, wetting of polluted soil with water to maintain its humidity appeared to be one of the main factors favoring the oil elimination.

Like in other works [8, 9, 24], the tested WRB usually secreted appreciated levels of LME in the presence of petroleum hydrocarbons. Especially high laccase activity was observed in the bioremediation process whereas low or traces of MnP and LiP activities were detected at the same time. In another study, *Daedaleopsis* sp. produced laccase, MnP, and LiP in the presence of crude oil and the authors concluded that elevated ligninolytic enzymes' production could be indicative of these enzymes' role in oil hydrocarbon degradation [8]. However, an analysis of the data received did not show any direct relationship between fungal enzyme activity and the degree of oil elimination. It is possible that other enzymes catalyze the biodegradation process [24], although it is not excluded that the time of enzyme activity measurement in this study did not coincide with the maximum laccase or peroxidases activity.

In conclusion, our results show that crude oil can be degraded by wide range of WRB in the submerged and solid-state fermentation conditions. However, the tested fungi significantly differ in their potential to eliminate pollutant during the bioremediation process. Usage of fungal mycelium pre-grown on a mixture of wheat straw and mandarin peels for inoculation of an artificially oil-polluted soil is an appropriate strategy. Supplementation of lignocellulosic material to the oil-polluted soil helps the fungi to rapidly colonize soil, form extensive mycelial networks using pollutant as a growth substrate and promotes production of lignin-modifying enzymes. The maximum degradation rate (65%) was obtained when *C. unicolor* 305 was incubated in the 1% oil-containing soil supplemented with a mixture of wheat straw and mandarin peels (10% of the soil weight) for 28 days. At the same conditions, *P. ostreatus* 2175 eliminated 43.9% of initial oil when its concentration in the soil was increased to 2%. Thus, the capability of these fungi to degrade crude

oil can be applied for the bioremediation of polluted environments.

ACKNOWLEDGMENTS: *This work was supported by the Shota Rustaveli National Science Foundation (project MTCU/63/7-280/15) and the Science and Technology Center in Ukraine (project 5629).*

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БИОДЕГРАДАЦИЯ НА СУРОВ НЕФТ И АКТИВНОСТ НА ЛИГНИН-МОДИФИЦИРАЩИ ЕНЗИМИ В БАЗИДИОМИЦЕТНИ ГЪБИ НА БЯЛОТО ГНИЕНЕ

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Резюме. В настоящата работа е изследвана способността на осемнадесет базидиомицетни гъби, предизвикващи бялото дървесно гниене, да деградират суров нефт. Щамове на *Cerrena unicolor*, *Panus tigrinus* 433, *P. lecometei* 903, *Pleurotus ostreatus* 70, *Trametes maxima* 403 и *T. versicolor* 159 показаха особено обилен растеж на повърхността на агар, покрит с капки нефт. Пълното обезцветяване на индикатора Резазурин беше постигнато в условията на дълбочинно култивиране на *Bjerkandera adusta* 139, *C. unicolor* 303, *Coriolopsis gallica* 142, *P. ostreatus* 70, *P. pulmonarius* 148 и *T. versicolor* 159 в продължение на две седмици в среда, съдържаща 3 г/л глюкоза. Максимално разграждане на нефта (65%), беше постигнато при инокулирането на изкуствено замърсена с нефт (1%) почва с гъбен мицел на *C. unicolor* 305, предварително отгледан на смес от пшенична слама и кори на мандарини в продължение на 28 дни. В същите условия *P. ostreatus* 2175 елиминира 43.9% от нефта, чиято концентрация в пръстта е 2%. Добавянето на глюкоза или дрождев екстракт към пръстта, съдържаща лигноцелулоза не усилва деградацията на нефта, докато поддържането на влажността на почвата с дестилирана вода благоприятства отстраняването на замърсителя. Изпитваните гъби секретират лигнин-модифициращи ензими в присъствие на нефт: колкото по-високо бе съдържанието на лигноцелулоза в инокулираната почва, толкова по-висока беше ензимната активност на гъбите. Получените резултати обаче не свидетелстват за пряка корелация между ензимната активност и степента на деградация на нефта.

Ключови думи: микоремедиация, суров нефт, базидиомицети, условия на култивиране, лигнин-модифициращи ензими

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