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RESEARCH ARTICLE

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Degradation of High Concentrations of Anthracene Using White-Rot Wood-Inhabiting Fungi and Investigation of Enzyme Activities

Mohadeseh Khajehzadeh[a], Masoomeh Ghobad-Nejhad[a], Hamid Moghimi[b], Ali Abolhasani Soorki[c], Yu-Cheng Dai[d] and Jing Si[d]

[a]Department of Biotechnology, Iranian Research Organization for Science and Technology (IROST), Tehran, Iran; [b]Department of Microbiology, School of Biology, College of Science, University of Tehran, Tehran, Iran; [c]Department of Petroleum Microbiology, Research Institute of Applied Sciences, ACECR, Shahid Beheshti University, Tehran, Iran; [d]State Key Laboratory of Efficient Production of Forest Resources, School of Ecology and Nature Conservation, Beijing Forestry University, Beijing, China

ABSTRACT

Owing to the production of lignin-modifying enzymes (LMEs), white-rot fungi (WRF) such as polypores are potent organisms in the biodegradation of xenobiotic pollutants. The nonspecific function of LMEs including laccase and manganese peroxidase (MnP), has enabled the use of WRF in biotechnological applications, particularly in bioremediation. In this study, 12 strains from nine white-rot basidiomycete genera viz., *Ganoderma*, *Inocutis*, *Irpex*, *Lentinus*, *Lenzites*, *Oxyporus*, *Peniophora*, *Sanghuangporus*, and *Trametes* were isolated from Iran and identified using morphological and molecular tools. The enzyme activity of laccase and manganese peroxidase that directly correlated with the biodegradation were determined, and the strains with the highest enzyme activities were evaluated for their ability to degrade 400 mg/L of anthracene over 28 days. Gas chromatography with flame ionization detector (GC-FID) revealed that four polypores viz., *Trametes versicolor* v21te, *T. versicolor* v22da, *T. hirsuta*, and *Oxyporus* sp. degraded 64%, 52%, 34%, and 20% of the anthracene, respectively. According to our analysis, the examined indigenous WRF are potentially useful candidates for the development of new mycoremediation techniques to degrade polycyclic aromatic hydrocarbons (PAHs).

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1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are hydrophobic substances found in the petroleum, gas production, and wood processing industries [1, 2]. They pose significant environmental and human health risks owing to their limited water-dissolution ability.

Various diseases, including asthma, anemia, splenomegaly, and different types of cancer have been attributed to PAHs and their derivatives [3]. In addition, the effect of some low molecular weight PAHs on reproductive abnormalities and even death of aquatic animals has been proven [4]. Anthracene is a low molecular weight micropollutant that is considered as a representative of the PAH group [5].

Bioremediation is a promising PAH remediation technique that uses various organisms that able to degrade this king of pollutants [6–8]. Mycoremediation, an efficient method among various bioremediation strategies, has gained significant attention in recent years, as it is an efficient method that has been used to degrade pollutants through the biodegradative capabilities of fungi [8–11]. White-rot fungi (WRF) are a physiological group of basidiomycetes that, because of their production of lignin-modifying enzymes (LMEs), including laccase and manganese peroxidase (MnP), play an essential role in the decomposition of lignin present in wood and in the biodegradation of a wide group of hazardous materials with aromatic structures [12, 13].

The low substrate specificity of LMEs resonates the unique ability of WRF, which has recently received much attention for biotechnological and bioremediation applications [13–15]. Creating an extensive hyphal network of spreading mycelia during growth, which facilitates their access to pollutants, has surprisingly enhanced their efficiency [16]. The degradation capability of WRF through the formation of PAH-quinones is the result of extracellular ligninolytic enzyme production [6, 17–20], which produces metabolites with less toxicity [15].

Anthracene, a tricyclic aromatic hydrocarbon pollutant is more soluble than other PAHs due to its low molecular weight, and therefore can contaminate water at significant levels [21]. Considering anthracene as a threat to the environment justifies the

CONTACT Masoomeh Ghobad-Nejhad ghobadnejhad@gmail.com; Hamid Moghimi hmoghimi@ut.ac.ir

importance of these studies [4, 22]. Anthracene degradation has been evaluated in several basidiomycetes, including *Bjerkandera* sp., *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *Trametes* sp., and *Irpex lacteus* [23–25]. However, the identification of new and potential WRF strains for PAH biodegradation, with special emphasis on their ligninolytic enzyme activity, remains a priority for mycologists [16, 26]. Furthermore, the use of indigenous Iranian basidiomycete fungi for enzyme production and pollutant remediation has not been investigated previously. In the present research, we aimed to evaluate indigenous wood-inhabiting basidiomycete strains in producing ligninolytic enzymes and degrading anthracene.

2. Materials and methods

2.1. Isolation of fungal strains

The fresh basidiomata were collected from various habitats in Iran during 2021-2022 and immediately grown on potato dextrose agar (PDA) to obtain mycelial cultures. Morphological identification of the basidiomata was performed by Masoomeh Ghobad-Nejhad based on macroscopy and microscopy characteristics. Most of the isolates were obtained from lowland temperate forests in Semnan Province (35.2256°N, 54.4342°E). To determine optimal growth conditions, fungal strains were cultured at temperatures ranging from 25 to 37 °C and pH 4 to 7.

2.2. Molecular identification

Total genomic DNA of fungal mycelia was extracted with Favorgen nucleic acid extraction kit. The internal transcribed spacer region was amplified using the primer pair ITS1F and ITS4 following the protocol described in Ghobad-Nejhad et al. [27]. The newly obtained sequences were searched against the NCBI BLAST database [28] and were finally submitted to GenBank with the accession numbers reported in Table 1. The fungal isolates were deposited in the national collection of living fungi of Iran (acronym IRAN).

2.3. Enzymatic assays

Laccase is commonly used as an indicator to screen potent fungi for bioremediation applications. Therefore, ABTS and Guaiacol were used as substrates for laccase detection. PDA plates containing 3 mM ABTS and 4 mM of Guaiacol [29] were inoculated separately with the strains. The fungi were incubated at 28 °C in the dark for seven days. In the plate assay method, the color changes in the media are evaluated. The enzymatic activities of laccase and MnP were also quantitatively measured using the absorbance at 420 and 469 nm, respectively. The assays were performed using the supernatant obtained from the fungal cultural PDB medium filtrated through a 0.22 µm membrane. To measure laccase activity, 100 mM sodium acetate (pH 4.5) and 0.5 mM ABTS were used [18, 30]. To measure MnP activity, 50 mM sodium malonate (pH 4.5), 0.5 mM $MnSO_4$, 1 mM 2,6-dimethoxylphenol, and 0.1 mM H_2O_2 were used [31]. All assays were carried out in triplicate at room temperature for 28 days. Enzyme activity was expressed as units per liter (U/L), and one unit represents the amount of enzyme oxidizing one µmol of substrate per minute [32].

2.4. Evaluation of fungi in tolerance of anthracene

Potent fungi, selected by enzyme analysis, were evaluated for anthracene tolerance. The fungal isolates were cultured on PDA plates amended with ten different concentrations of anthracene from 100 to 1000 mg/L. Anthracene was dissolved in acetone and then added to the cooling agar solution. Each fungal culture was incubated in triplicate at 28 °C for 7 days. The concentration at which the fungi showed a normal

Table 1. LMEs Screening of isolated fungal strains in PDA medium supplemented with ABTS and guaiacol as indicators.

Strain	GenBank accession number	Qualitative assessment[a]		Quantitative assessment[b]	
		ABTS	Guaiacol	MnP (U/L)	Laccase (U/L)
Ganoderma australe	PP349772	+	+	5.2 ± 0.5	8.1 ± 0.9
Inocutis levis	PP349773	+	+	5.7 ± 0.3	10.4 ± 1.7
Inocutis tamaricis	PP349774	+	+	7.6 ± 0.3	9.7 ± 1.4
Irpex lacteus	PP349775	+	+	5.4 ± 0.6	9.1 ± 1.1
Lentinus tigrinus	PP349776	++	++	10.3 ± 0.8	30.6 ± 1.9
Lenzites warnieri	PP349777	+	+	3.9 ± 0.6	6.2 ± 0.4
Oxyporus sp.	PP349778	+++	+++	16.4 ± 1.7	45.8 ± 2.6
Peniophora quercina	PP349779	++	+	6.3 ± 0.7	7.4 ± 0.7
Sanghuangporus sp.	PP349780	+	+	4.1 ± 0.6	8.3 ± 0.8
Trametes hirsuta	PP349781	++++	++++	52.2 ± 2.1	60.9 ± 3.6
Trametes versicolor v21te	PP349782	++++	++++	54.9 ± 4.1	78.2 ± 5.1
Trametes versicolor v22da	PP349783	++++	++++	47.7 ± 3.5	70.6 ± 4.8
Control	–	–	–	0	0

[a]++++ high color change (up to 50 mm), +++ medium color change (up to 40 mm), ++ low color change (up to 25 mm), + negligible color change (up to 15 mm), and - no color change.
[b]Maximal enzyme activity of each fungus during the culture period based on U/L.

growth rate was selected for subsequent assessments [33].

2.5. Degradation of anthracene

The potentially selected strains were evaluated for anthracene biodegradation. Kirk's medium containing (g/l): glucose 10 g; KH_2PO_4, 2 g; $MgSO_4{\cdot}7H_2O$ 0.5 g; $CaCl_2$ 0.1 g; $MnSO_4$ 0.03 g; NaCl 0.06 g; $FeSO_4{\cdot}7H_2O$ 6 mg; $CoCl_2$ 6 mg; $ZnSO_4{\cdot}7H_2O$ 6 mg; $CuSO_4$ 6 mg; $AlK(SO_4)_2{\cdot}12H_2O$ 0.6 mg; H_3BO_3 0.6 mg; $Na_2MoO_4{\cdot}2H_2O$ 0.6 mg; yeast extract 0.012 g; diammonium tartarate ($C_4H_{12}N_2O_6$) 0.2 g; thiamin 1 mg; and Tween 80 0.5 g was used. The pH of the medium was adjusted to 4.5. All media were sterilized by autoclaving at 121 °C for 20 min [32, 34]. The experiments were conducted by inoculating five fungal agar plugs from the outer edge of the growing culture into 250 mL Erlenmeyer flasks containing 100 mL of Kirk's medium. Each fungal inoculum was prepared by culturing the selected fungus on a PDA plate at 28 °C for 7 days. To induce physiological preparation of fungal cells for bioremediation, each fungus was pre-cultured on PDA supplemented with 10% of the final concentration of the pollutant.

The liquid cultures were polluted by anthracene dissolved in acetone. The final anthracene concentration of each culture medium was 400 mg/L. The cultures were shaken at 140 rpm for 28 days at 28 °C. All treatments were replicated three times. The same procedure was performed for the control flasks, containing autoclaved inoculated and uninoculated media. Autoclaved inoculated media were investigated to elucidate whether the reduction of anthracene was due to biosorption or biodegradation. PAH analysis and enzyme assays were performed every seven days, for a total of 28 days [15].

2.6. Analysis of degredation with gas chromatography

To measure the remaining anthracene, the treatments were extracted with 100 mL of dichloromethane [35] and then concentrated to 5 mL using a vacuum rotary evaporator. The anthracene concentration was measured by injecting 1 µL of the extract into a Gas chromatography with flame ionization detector (GC-FID Shimadzu model GC-15A, Kyoto, Japan) equipped with a capillary column (30 m length and 0.25 mm diameter and 0.25 µm film thickness). The temperature program of the column was set at 80 °C, held for one min and then raised from 80 °C to 280 °C at a rate of 15 °C/min, and held for 5 min. The injector and detector temperatures were set to 280 °C. The flow rate was 1.5 mL/min, the injection volume was 1 µL, and the nitrogen pressure was 100 kPa. The residual anthracene in treated and control samples was compared to determine the effectiveness of fungi in bioremediation.

2.7. Statistical analysis

Minitab 17 package software was used to assess the experimental results. Data in the Table 1 were expressed as mean ± standard deviation (± SD). Differences between means were analyzed by the ANOVA test followed by the Tukey's test. *P*-values ($p < 0.05$) were used to measure the significance of the treatment effects.

3. Results and discussion

3.1. Fungal strains and LMEs production

Twelve strains belonging to nine basidiomycete genera *Ganoderma*, *Inocutis*, *Irpex*, *Lentinus*, *Lenzites*, *Oxyporus*, *Peniophora*, *Sanghuangporus*, and *Trametes* were isolated and identified in this study using morphological and molecular tools (Table 1).

The temperature of 28 °C and pH 4.5 were selected as optimal growth conditions for isolated fungi. LMEs screening of the fungal isolates was performed based on the plate assay method, in which enzyme-producing fungi were identified by the color change of the culture plates. The dark green color results from the oxidation of ABTS, whereas the dark brown color is due to the oxidation of Guaiacol [29]. Based on the size of the colored halo formed, *Trametes versicolor* v21te, *T. versicolor* v22da, *Trametes hirsuta*, and *Oxyporus* sp. showed the highest oxidative activity. The activity of *Lentinus tigrinus*, *Inocutis levis*, *I. tamaricis*, and *Peniophora quercina*, was low, and *Ganoderma australe*, *Sanghuangporus* sp., *I. lacteus*, and *Lenzites warnieri* showed a slight color change but much less than the others (Table 1). Similarly Vipotnik et al. [36] showed that WRF such as *T. versicolor* produce enzymes such as laccases, which can partially degrade PAH molecules. This enzyme plays a crucial role in bioremediation studies, as it initiates the breakdown of PAH molecules during degradation.

3.2. Enzyme activities

Laccase and MnP of the isolated fungi were evaluated for 28 days in the optimal conditions mentioned above. All assays were compared with a blank medium and a sterilized fungal medium as controls. Four potent fungi showed significant enzyme activity of laccase and MnP which reached its highest level

within two weeks and then decreased. The highest laccase and MnP activities were shown by *T. versicolor* v21te, *T. hirsuta*, *T. versicolor* v22da, *Oxyporus* sp., and to a lesser extent by *L. tigrinus*, respectively. *T. versicolor* v21te, *T. hirsuta*, and *T. versicolor* v22da showed the maximum laccase activity at the end of the second week with 78, 70, and 60 U/L respectively. The highest laccase activities of *Oxyporus* sp. and *L. tigrinus* were 44 and 30 U/L after 10 days of cultivation.

Trametes versicolor v21te, *T. hirsuta*, *T. versicolor* v22da, and *Oxyporus* sp. had the highest MnP activity in the first week. The peak of MnP activity in *Oxyporus* sp. was also at the end of the first week, with the difference that its activity was lower than that of the previously mentioned fungi. Partial MnP activity was observed in *L. tigrinus* at approximately 10 U/L.

Peniophora quercina and *I. levis* showed trifle production of both enzymes after 14 days, but this was not remarkable. Although laccase and MnP activity was detected in *L. warnieri*, *I. lacteus*, *G. austral*, *I. tamaricis*, and *Sanghuangporus* sp., it was negligible compared to *T. versicolor* v21te, *T. versicolor* v22da, *Oxyporus* sp., and *T. hirsuta*. The activity pattern of laccase and MnP in the selected fungi of this study was consistent with *Rigidoporus* sp. [16].

The main mechanism for biodegrading ability of WRF is the production of nonspecific ligninolytic enzymes [8, 20, 37, 38]. Only a few fungi with ligninolytic enzyme activity have been studied as potential agents for bioremediation, leaving many species unexplored [20].

In the present research, ligninolytic enzyme activity was investigated in both the presence and absence of anthracene. Out of the 12 polypores examined here, to the best of our knowledge, *Inocutis levis*, *I. tamaricis*, *Lenzites warnieri*, and *P. quercina*, as well as the genera *Sanghuangporus* have not been previously evaluated for their ligninolytic enzyme capabilities and bioremediation potential. The mycoremediation activities of *T. versicolor* [23], *T. hirsuta* [39], and *I. lacteus* [40] have been studied before.

3.3. Assessment of anthracene degradation

Our analyses showed that the four potent fungi, *T. versicolor* v21te, *T. versicolor* v22da, *T. hirsuta*, and *Oxyporus* sp. can tolerate up to 400 mg/L of anthracene. The degradation process began in the first week and reached its maximum after 28 days (Figure 2). The biodegradation ability varied for different fungi. Both inoculated and non-inoculated control samples showed a 10% reduction in anthracene after 28 days, indicating that fungal absorption had not occurred.

The enzyme activity of the strains during anthracene degradation is shown in Figure 1. The laccase activity in *T. versicolor* v21te, *T. versicolor* v22da, *T. hirsuta*, and *Oxyporus* sp. has increased from 78, 70, 60, and 44 U/L to 190, 210, 180, and 150 U/L, respectively. Moreover, the MnP activity in *T. versicolor* v21te, *T. versicolor* v22da, *T. hirsuta*, and *Oxyporus* sp. has increased from 54, 47, 52, and 16 U/L to 114, 90, 105, and 50 U/L, respectively. Laccase and MnP activity increased significantly in the presence of anthracene, indicating a direct correlation between enzyme activity and pollutant degradation.

Anthracene caused a 3-fold increase in laccase activity in all four fungi cultured in Kirk's medium. The enzyme activity was maintained until the end of the third week. The highest activity of MnP in the medium without pollutants was at the end of the first week, after which the downward trend of enzyme activity started immediately. The presence of anthracene resulted in a two-fold increase in MnP activity, which continued until the third week.

The results strongly show that in the presence of anthracene, the reduction rate of enzyme activity occurs more slowly and laccase activity was significant after 28 days. Acevedo et al. [41] found that

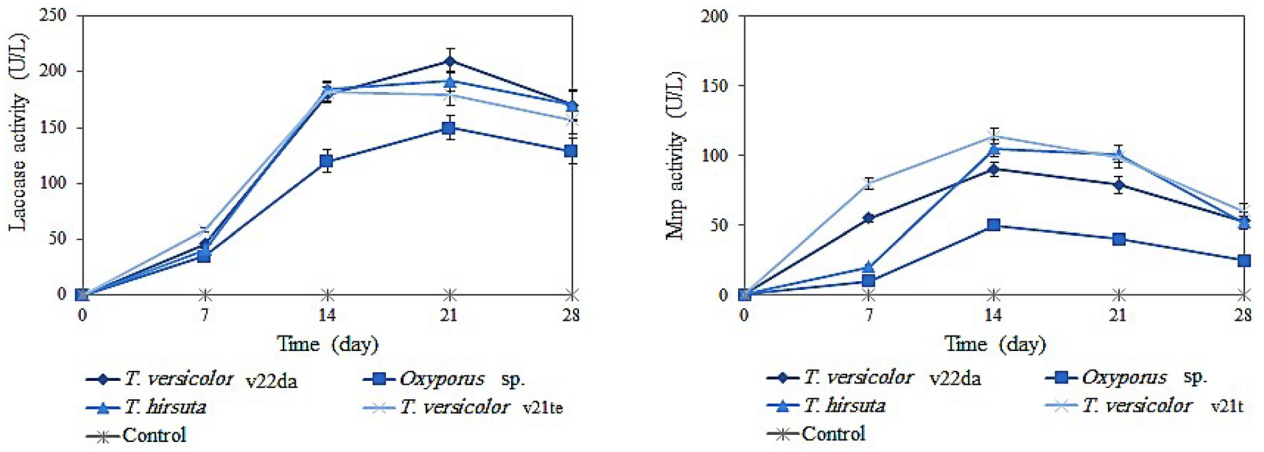

Figure 1. Dynamics of laccase and MnP activities during anthracene degradation.

PAHs stimulates the synthesis of MnP in *Anthracophyllum discolor*, leading to a reduction in PAH concentration. Using the strong oxidizing agent Mn^{3+}, MnP can effectively break down PAHs, with ionization potentials of up to 7.8 eV such as anthracene.

The peak activity of MnP and laccase is not at the same time, which can be considered an advantage for their cooperation in bioremediation, as it has been pointed out in other studies [16, 42].

It is worth mentioning that a slow downward trend in laccase activity over 28 days would be beneficial for optimal biodegradation. According to Figure 2, the highest percentage of pollutant degradation occurred during the second and third weeks, which coincided with the peak of the enzymatic activities of the fungi. Therefore, enzyme activities and pollutant degradation are strongly correlated [37, 40, 43]. In fact, pollutants can promote bioremediation by inducing enzyme production [20, 30]. During stressful conditions, such as exposure to pollutants, fungi produce ligninolytic enzymes as a defense mechanism which leads to bioremediation [14].

Many researchers have pointed out the effect of ligninolytic enzymes on the biotransformation pathway of anthracene, but there are limited reports on the degradation pathway of anthracene [37, 44]. According to the studies, the path of anthracene degradation can be different. In one of the valid proposed pathways, ligninolytic enzymes first oxidize *C*9 and *C*10 of anthracene molecules, then by ring cleavage, the anthracene is opened, and compounds such as phthalic acid is produced. The product of the next step will be benzoic acid, which will finally turn into catechol. During the process, intermediate metabolites such as phthalic acid and anthrone are quickly converted into subsequent metabolites and eliminated

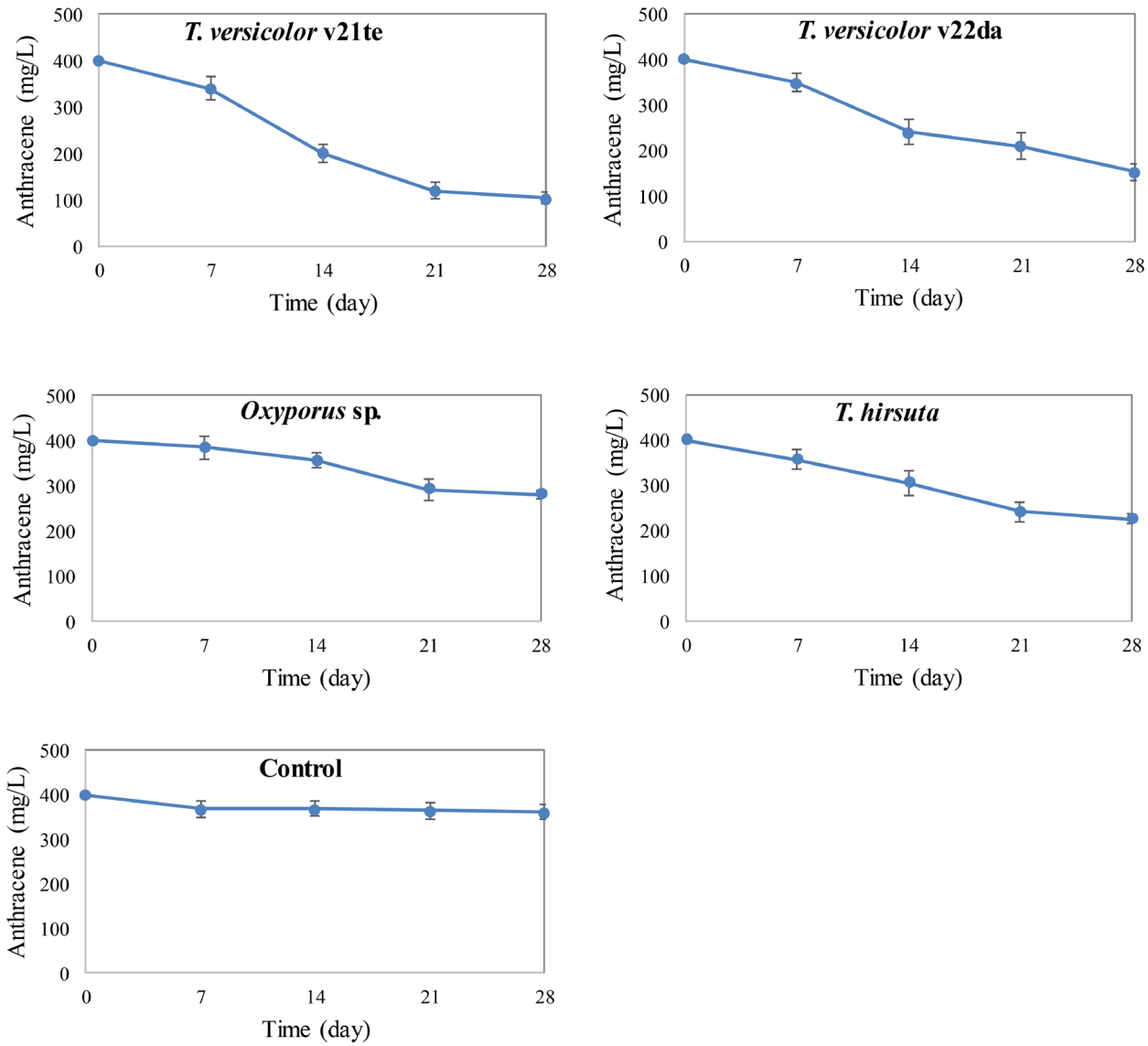

Figure 2. The degradation of 400 mg/L anthracene by *Trametes versicolor* v21te, *T. versicolor* v22da, *T. hirsuta*, and *Oxyporus* sp. Error bars indicate standard deviation.

[37, 45]. The oxidation pathway of anthracene may differ from one organism to another, depending on the type of enzymes produced. Wu et al. have also introduced anthraquinones as the main product of laccase oxidation [46]. In general, the ligninolytic enzymes of WRF increase the bioavailability of PAHs by producing PAH-quinones, which usually have a higher solubility and less toxicity than their parent compounds [47]. This suggests their development and use for the biodegradation of anthracene.

In the present study, our native isolates of *T. versicolor* v21te, *T. versicolor* v22da, *T. hirsuta*, and *Oxyporus* sp. demonstrated the ability to grow and produce enzymes in 400 mg/L anthracene. Previous studies have shown biodegradation of lower concentrations of anthracene. Depending on the type of strains, concentration of the pollutant, culture medium, and treatment period, different fungi have shown different capabilities in the biodegradation of pollutants (Table 2). For example, *P. chrysosporium*, *T. versicolor*, and *Bjerkandera adusta* have shown significant results in 10 mg/L of anthracene biodegradation, So that they have exhibited the ability to decompose more than 80% of this pollutant [25]. Degradation efficiency of 1 mM anthracene by *Polyporus* sp. S133 is reported to be 92% within 30 days. The highest enzyme activity is related to day 15, which aligns with our results [44]. Complete removal of 1 mg/L of anthracene using immobilized *P. chrysosporium* cells within 7 days has been reported by Mohammadi and Nasernejad [5]. The removal of 50 mg/kg of anthracene by *P. chrysosporium*, *T. versicolor*, and *Pleurotus ostreatus* has been successful. *P. ostreatus* had more biodegradation capability compared to the other fungi [40]. In another study, the ability of *P. ostreatus* to remove 33-40% of tricyclic PAH such as anthracene from contaminated soil with 50 mg/kg of anthracene was observed [24]. Furthermore, the removal of 15%, 38%, and 40% of 100 mM anthracene has been reported by *P. ostreatus*, *I. lacteus*, and *P. chrysosporium*, respectively. *P. ostreatus* was introduced as a relatively poor strain for biodegradation [3]. While another study concluded the opposite [1]. These examples show the different capabilities of fungi in anthracene degradation. Five *Peniophora incarnata* strains and two *P. sordida* strains respectively have shown the ability to degrade 40-70% and up to 80% of 30 mg/L of anthracene in combination with pyrene, fluoranthene, and phenanthrene (final concentration of mixture: 120 mg/L) [30].

According to the mentioned examples, the ability of white rot fungi to degrade PAH is obvious, but most important is the exceptional ability of our indigenous fungi, which can degrade a significant percentage of it in addition to growth and enzyme activity in the presence of 400 mg/L of anthracene. These results provide a suitable approach for industrial applications. In most cases, the concentration of anthracene in the environment is higher than these levels, particularly in areas exposed to petroleum and gaseous products [2]. Hence, for the field use of these fungi and their enzymes, it is necessary to focus on strategic methods such as identifying potent strains for biodegradation of high concentrations of the pollutants. This is the first study that investigates the indigenous strains of Iran and evaluates their enzyme activity and the ability to remove high concentrations of anthracene.

Table 2. Comparison of anthracene degradation by white rot fungi.

Strain	Initial anthracene concentration	Treatment period (day)	Removal (%)	Reference
Trametes versicolor v21te	400 mg/L	28	64	This study
Trametes versicolor v22da	400 mg/L	28	52	This study
Trametes hirsuta	400 mg/L	28	34	This study
Oxyporus sp.	400 mg/L	28	20	This study
Phanerochaete chrysosporium	10 mg/L	28	92.6	[25]
Trametes versicolor	10 mg/L	28	80	[25]
Bjerkandera adusta	10 mg/L	28	99.2	[25]
Polyporus sp. S133	1 mM	30	92	[44]
Phanerochaete chrysosporium	1 mg/L	7	100	[5]
Phanerochaete chrysosporium	50 mg/kg	56	0.6	[40]
Trametes versicolor	50 mg/kg	56	0.6	[40]
Pleurotus ostreatus	50 mg/kg	56	34.8	[40]
Pleurotus ostreatus	50-200 mg/kg	120	33-45	[24]
Pleurotus ostreatus	100 mM	61	15	[3]
Irpex lacteus	100 mM	61	38	[3]
Phanerochaete chrysosporium	100 mM	61	40	[3]
Peniophora incarnata	40 mg/L	14	40-70	[30]
Phanerochaete sordida	40 mg/L	14	up to 80	[30]

4. Conclusions

In the present study, a number of Iranian native WRF were investigated for the first time in terms of ligninolytic enzyme activities and bioremediation. The isolated fungi including *T. versicolor* v21te, *T. versicolor* v22da, *T. hirsuta*, and *Oxyporus* sp. were able to produce laccase and MnP and degrade 400 mg/L anthracene. The biodegradation of anthracene depended on the enzymatic activities of the fungus, and those with more laccase and MnP activities showed greater bioremediation potential. According to the results, we managed to widen the spectrum of our fungal choice and examined the potential of additional fungal genera and species for LMEs enzyme production and pollutant degradation. This would render the opportunity to dig into the wealth of natural resources of fungal diversity to find innovative solutions for the human-made threats to the environment.

Author contributions

Conceptualization and methodology, MG, HM and MK; investigation and formal analysis, MK; resources, MG and AA; data curation, MK, HM and MG; validation, HM, MG, JS, YCD; writing—original draft preparation, MK and MG; writing—review and editing, MK, MG, HM, AAS, JS, and YCD; visualization, MK; supervision, MG and HM; project administration, MG; funding acquisition, MG. All authors have read and agreed to the published version of the manuscript.

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ORCID

Mohadeseh Khajehzadeh http://orcid.org/0009-0004-1643-2606
Masoomeh Ghobad-Nejhad http://orcid.org/0000-0002-7807-4187
Hamid Moghimi http://orcid.org/0000-0002-9454-7474
Ali Abolhasani Soorki http://orcid.org/0000-0002-4952-5567
Yu-Cheng Dai http://orcid.org/0000-0002-6523-0320
Jing Si http://orcid.org/0000-0001-9229-0727

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