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Full Length Research Paper

Biodegradation of some polycyclic aromatic hydrocarbons by *Aspergillus terreus*

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In our search for new fungal isolates capable of degrading polycyclic aromatic hydrocarbons (PAHs) in soil, twenty one fungal isolates were recovered from Orman Garden, Wadi Degla Protectorate and benzene station soils. All tested fungi exhibited lignin peroxidase and manganese peroxidase activities in solid as well as in liquid cultures. However, laccase was detected in low amounts by some of the tested fungal isolates. Accordingly, laccase was eliminated from further work. *Aspergillus terreus* was superior in ligninolytic enzyme production. Hence, it was chosen for the following studies. The statistical optimum temperatures for lignin peroxidase and manganese peroxidase production by *A. terreus* were 33.6 and 33.1°C, respectively. Meanwhile lignin peroxidase and manganese peroxidase yields were maximal at pH 4.1 and 5.8, respectively. Highest ligninolytic enzyme secretions were established on D-glucose and sodium nitrate. An experiment to study biodegradation of PAHs in soil was conducted. *A. terreus* was able to degrade 98.5% of naphthalene and 91% of anthracene in soil models.

Key words: Biodegradation, fungi, polycyclic aromatic hydrocarbons (PAHs) Ligninolytic enzymes, soil model.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are fused ring aromatic compounds, ubiquitous pollutants in the atmosphere and relatively resistant to biodegradation because of their hydrophobicity (Antizar-Ladislao et al., 2006). PAHs can therefore accumulate to substantial levels in the environment and have been detected in a wide range of soils and sediments, including some ancient sediment (Fernandez et al., 1992). There are more than 70 compounds classed as PAHs having from 2 to 7 rings. Since the larger compounds are carcinogenic (Yu, 2002), they can pose a significant health hazard.

Although PAHs consist of fused benzene rings, lignin is a much larger, more heterogeneous and amorphous polymer made up of phenylpropane subunits with a high content of benzene ring-oxygen, ether linkages and abundant carbon–carbon side chains, that is why ligninolytic enzymes can attack PAHs (Gadd, 2001).

Three major classes of extracellular enzymes designated manganese- dependent peroxidases (MnPs), lignin peroxidases (LiPs), and laccases are believed to be important in the fungal degradation of lignin (Silva et al., 2010).

Chemical, physical as well as biological methods are used for PAHs remediation. Above all, biological methods are favored because of their good results and low costs (Wu et al., 2010). Microbial biodegradation is a friendly and effective means to remove polycyclic aromatic hydrocarbons (PAHs) from the environment and has been extensively used (Wanga et al., 2010).

This work aimed to screen the ligninolytic activity of some fungal isolates. More consideration was given to some cultural conditions influencing production of ligninolytic enzymes by the most potent fungus. The aim was extended to determine the degree of PAHs`

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degradation in a soil model.

MATERIALS AND METHODS

For isolation of biodegrading fungi, soil samples were collected from three different sites [Orman Garden (Giza), Wadi Degla protectorate (Maady), and a bezene station (Giza) in Egypt]. Fungal isolation was carried out according to Johnson et al. (1960) using soil dilution plate method. A Czapek-Dox agar medium was used for such purpose. This medium contained the following (g/l): sucrose, 20; $NaNO_3$, 2; K_2HPO_4, 1; KCl, 0.5; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.01 and agar, 15, Streptomycin (30 µg/ml) was added to the above medium after sterilization by autoclaving at 121°C and 1.5 bars for 15 min.

Qualitative screening for ligninolytic enzyme activities

Czapek-Dox agar medium was employed for the screening of ligninolytic enzymes activities. 0.0025% azure B (w/v) or 0.0025% phenol red (w/v) were used to detect lignin peroxidase (Archibald and Roy, 1992) or manganese peroxidase (Kuwahara et al., 1984), respectively. Laccase was tested by adding 0.05% (w/v) guaiacol, modified from Kiiskinen and Saloheimo (2004). Fungal discs (1 cm in diameter) were taken from the periphery of 7-day old cultures grown on Czapek-Dox agar plates. Discs were inoculated onto triplicate plates containing the screening media. Plates were incubated at 30°C for 7 days in a static incubator. The diameter of the colored zone produced from chromogenic substrate metabolism was measured.

Quantitative estimation of ligninolytic enzyme activities

Triplicate flasks containing Czapek-Dox broth medium were inoculated with 1 cm diameter fungal discs taken from the periphery of 7 day old cultures grown on Czapek-Dox agar plates. The flasks were incubated at 30°C for 10 days. The culture filtrate was used for measuring the extracellular ligninolytic enzyme activities.

Following the methodology of Tien and Kirk (1988), lignin peroxidase activity was determined by monitoring the oxidation of veratryl alcohol to veratraldehyde at 37°C as indicated by an increase in absorbance at 310 nm (εmax = 9300 M-1 cm-1). The reaction mixture (2.5 ml) contained 500 µl enzyme extract, 500 µl (2 mmol/l) H2O2, 500 µl veratryl alcohol solution (10 mmol/l) and 1.0 ml sodium tartarate buffer pH 3.0 (10 mmol/l). One unit of enzyme activity is defined as the amount of enzyme oxidizing 1 µmol of substrate per minute.

Manganese-dependent activity was determined by measuring the increase of Mn^{+3}malonate formation at 270 nm (εmax =11,590 M^{-1} cm^{-1}) at 25°C. The assay mixture (1 ml) contained sodium malonate buffer (70 mM, pH 4.5), $MnSO_4$ (2 mM), H_2O_2 (6 mM), and 25 µl of enzyme extract. One unit of MnP was defined as 1 µmol of product formed per milliliter per minute under the assay conditions (Ledwozyw et al., 1986).

Laccase activity was assayed with guaiacol as substrate. The reaction mixture contained 3.9 ml acetate buffer (10 mmol/l, pH 5.0), 1 ml guaiacol (2 mmol/L) and 0.1ml properly diluted enzyme solution was incubated at 50°C for 30 min. Absorbance was read at 470 nm (εmax = 6,740 Mol^{-1} cm^{-1}). In the blank, guaiacol was replaced with acetate buffer (Das et al., 2008).

Enzymes activities are expressed in µmol of substrate oxidized per milliliter per minute, in the following equation: U ml^{-1} min^{-1} = ∆Abs (10^6) $(\varepsilon RT)^{-1}$, where ∆Abs is the difference between final and initial absorbances, ε is the extinction coefficient of each enzyme product (LiP - ε310 = 9300 mol^{-1} cm^{-1}; MnP-ε270 = 11,590 mol^{-1} cm^{-1}; Lac –ε450 = 65 000 mol^{-1} cm^{-1}), R is the volume in milliliters of supernatant, and T is the reaction time in minutes.

Effect of some physical and chemical factors on production of extracellular ligninolytic enzymes

In each case, lignin peroxidase and manganese peroxidase were measured in culture filtrate of *A. terreus*.

Incubation temperature

Triplicate flasks containing the previously described Czapek-Dox broth medium were prepared. The media were inoculated with fungal discs. Incubation was carried out at the following temperatures: 15, 20, 30, 40 or 50°C for 10 days in a static incubator. A quadratic regression model fit was conducted to find the optimum temperature.

pH

The following buffer solutions were used in Czapek-Dox broth medium: acetate buffer solution for pHs 3, 4 or 5; citrate buffer solution for pHs 4 or 5; and phosphate buffer solution for pHs 6, 7 or 8, each at a concentration of 0.1 M. Triplicate flasks were inoculated with fungal discs and incubated at 31°C (the best incubation temperature) for 10 days. The optimum pH was measured using a quadratic regression model fit.

Carbon source

Sucrose was used or replaced in Czapek-Dox broth medium by: D-glucose, sucrose, lactose, starch or maltose.

Nitrogen source

The liquid medium containing 20 g/l of the chosen carbon source from the previous experiment was used as a control medium with 2 g/l $NaNO_3$. Sodium nitrate was replaced by the following inorganic nitrogen sources like 1.9 g/l ammonium nitrate, 2.4 g/l potassium nitrate, and organic nitrogen sources like 1.8 g/l glycine, and 2.64 g/l peptone. The nitrogen sources were used at equimolecular weights of nitrogen.

Analysis of biodegradation of PAHs in a soil model

To study the ability of *A. terreus* for biodegradation of PAHs (naphthalene or anthracene), 5 g sterilized soil samples were used. Soil sterilization was performed by autoclaving three times at 121°C for 45 min each over three consecutive days.

Soil samples were spiked with naphthalene or anthracene dissolved in acetone (150 ppm final concentration), homogenized, and incubated at room temperature (about 30°C) overnight to allow evaporation of acetone. *A. terreus* (grown on sorghum seed for two weeks before) was then transferred to the naphthalene or anthracene -spiked soil and then incubated at room temperature (about 30°C). The residual level of PAH was determined by HPLC every week for four weeks. PAH was extracted with 10 ml dichloromethane, and vortexed. The dichloromethane phase was transferred to a test tube and the solvent was removed with a vacuum evaporator, leaving residual PAH, which was reconstituted in 1 ml methanol (HPLC grade) and subjected to HPLC for product identification.

To ensure the accuracy of extraction, an internal standard of 150

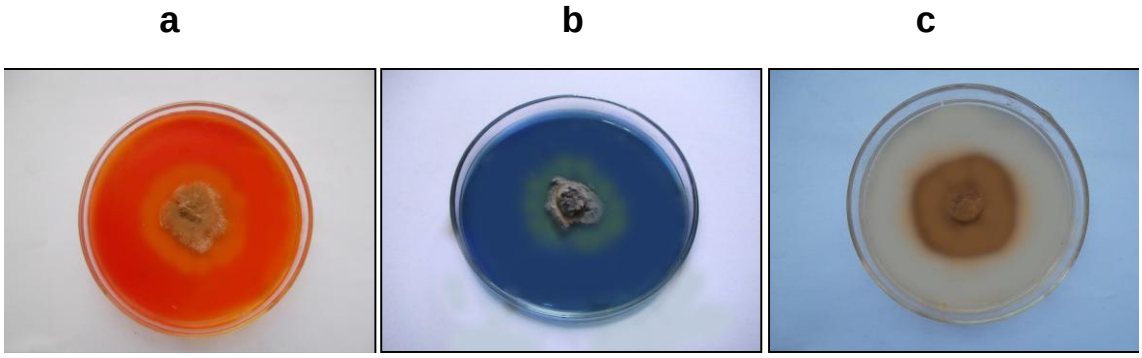

Figure 1. Ligninolytic enzymes production in solid media by *A. terreus* isolated from Orman Garden. a: oxidation of phenol red to form yellow zones. b: oxidation of azure B to form green zones. c: oxidative polymerization of guaiacol to form reddish brown zones.

ppm naphthalene or anthracene solutions was spiked into replicate samples before extraction and thereafter treated in the same manner as the experimental samples (Chupungars et al., 2009).

PAHs were quantified by a Shimadzu high-performance liquid chromatography (HPLC) system equipped with a model SCL10AVP system controller, SCL10AVP pumps, a SIL 10ADVP automatic injector, and a SPD10APV UV detector (Shimadzu Corp., Kyoto, Japan). The column used was an Inertsil ODS-3, a reverse phase C18. The mobile phase was 80:20 (v/v) methanol:water at a flow rate of 1 ml min^{-1}.

The presence of PAHs and their respective metabolites was monitored at 254 nm using HPLC. The mobile phase was set to 45 min linear gradient of methanol–water (from 40:60 to 80:20 [v/v]) for both separation and analysis of metabolites. Identification of PAHs and its metabolites was carried out by comparing their relative retention times with those of standards.

Statistical analysis

Data presented in each experiment were means of triplicate assays. The SPSS 16.0 software was used in determination of standard error (SE), least significant difference (LSD) and regression analysis.

RESULTS AND DISCUSSION

Fungi, including saprophytic, plant root-parasitic fungi and wood decaying fungi, have been shown to degrade PAHs, indicating their potential as environmental bioremediators of these compounds (Silva et al., 2009). In this work, such fungi were expected to occur in Orman Garden and Wadi Degla protectorate soils, as well as the benzene station soil contaminated with oil residues. Twenty one fungal isolates were recovered from the three sites, seven isolates from each source. To the wood-decaying fungi, the average rates of PAH conversion have been correlated with average activities of ligninolytic enzymes (Eibes et al., 2006).

In the current study, it was found that all fungi tested could produce lignin peroxidase (Table 1). *A. terreus* isolated from Orman Garden soil achieved the highest statistically significant colored zone (6.2 mm) after 10 days. It was followed by *Penicillium chrysogenum* isolated from Wadi Degla protectorate soil (4.07 mm). Figure 1a shows the oxidation of phenol red to form yellow zones in the medium by *A. terreus* isolated from Orman Garden.

All fungi tested in the current study were capable of producing manganese peroxidase (Table 1). Once more, *A. terreus* isolated from Orman Garden protectorate soil achieved the highest statistically significant colored zone (20.0 mm). It was followed by *Aspergillus fumigatus* isolated from Orman garden protectorate soil (13.0 mm).The oxidation of azure B to form green zones in the medium by *A. terreus* isolated from Orman Garden is shown in Figure 1b.

It is noteworthy that not all 21 tested fungi exhibited laccase activity (Table 1). Only eight isolates showed laccase activity. However, *A. terreus* isolated from Orman Garden soil again achieved the highest statistically significant reddish brown zone (17.03 mm). Figure 1c shows oxidative polymerization of guaiacol to form reddish brown zones in the medium by *A. terreus* isolated from Orman garden.

The quantitative estimation of lignin peroxidase in the present results (Table 2) revealed that both *A. terreus* isolated from Orman Garden soil and *P. chrysogenum* isolated from Wadi Degla protectorate soil achieved the highest statistically significant activities (3.0198 and 3.0757 U/ml, respectively). The detected activities in this study are higher than those obtained by Kapich et al. (2004) for *Phanerochaete chrysosporium* ME-446 (0.32 U/ml) and Hadibarata et al. (2009) for *Polyporus* spp. S133 (0.06 U/ml), but lower than that by *Mucor racemosus* CBMAI 847 (75.376 U/ml) as reported by Bonugli-Santos et al. (2010) .

It appears from Table 2 that the potent *A. terreus* isolated from Orman Garden soil achieved the highest statistically significant manganese peroxidase activity (20.2194 U/ml). *A. fumigatus* isolated from Orman Garden soil (13.2427 U/ml) followed. The manganese peroxidase activities reached by soil fungi in this work are

Table 1. Plate assay for ligninolytic enzymes of the isolated fungi. Data are expressed as diameter of colored zone (mm) after 7 days of incubation at 30°C.

Fungal species	Laccase	Mn Peroxidase	Lignin peroxidase
Wadi Degla protectorate			
Alternaria alternata	0.00^{H}	2.53^{M}	3.4^{E}
Aspergillus flavus	9.90^{D}	3.47^{K}	3.5^{D}
Aspergillus fumigatus	5.10^{F}	1.00^{O}	2.2^{K}
Aspergillus ustus	0.00^{H}	12.53^{C}	2.1^{L}
Fusarium moniliforme	15.10^{B}	4.53^{I}	2.2^{K}
Fusarium pallidoroseum	0.00^{H}	3.00^{L}	3.2^{F}
Penicillium chrysogenum	4.80^{G}	3.50^{K}	4.07^{B}
Orman Garden soil			
Aspergillus aculeatus	0.00^{H}	9.00^{E}	2.5^{I}
Aspergillus flavus	0.00^{H}	8.00^{F}	2.7^{G}
Aspergillus fumigatus	4.03^{G}	13.0^{B}	3.6^{C}
Aspergillus terreus	17.03^{A}	20.0^{A}	6.2^{A}
Botryotrichum piluliferum	0.0^{H}	2.00^{N}	2.0^{M}
Cochliobolus lunatus	8.17^{E}	12.0^{D}	2.6^{H}
Emericella nidulans	0.0^{H}	3.00^{L}	2.3^{J}
Benzene Station soil			
Aspergillus aculeatus	0.0^{H}	5.00^{H}	2.7^{G}
Aspergillus flavus	0.0^{H}	4.00J	3.6^{C}
Aspergillus fumigatus	0.0^{H}	7.00^{G}	1.9^{N}
Aspergillus niger	0.0^{H}	2.00^{N}	3.6^{C}
Aspergillus terreus	14.00^{C}	2.00^{N}	3.6^{C}
Fusarium pallidoroseum	0.0^{H}	3.00^{L}	1.9^{N}
Gibberlla accuminata	0.0^{H}	3.00^{L}	2.3^{J}
LSD (at 0.01)	0.113	0.052	0.026

The least significant difference (LSD) was calculated at 99% confidence interval (probability of error p=0.01). Means followed by the same letters are statistically non significant.

obviously higher than those in previous reports. *M.*) *racemosus* CBMAI 847 (Bonugli-Santos et al., 2010) produced manganese peroxidase with activity 2.6 U/ml, while *P. chrysosporium* (Wen et al., 2010) showed an activity of 0.588 U/ml.

It is worthy to mention that laccase activity in the current work was detectable in only five isolates out of twenty one tested fungi and with very low values (Table 2). Accordingly, only manganese peroxidase and lignin peroxidase were further considered. In this study, *A. terreus* exhibited the highest significant lignin peroxidase as well as manganese peroxidase activities, both qualitatively and quantitatively. It was thus selected for the next studies.

Effect of some physical and chemical factors on production of ligninolytic enzymes by *A. terreus*

Temperature

The present results show that the statistical optimum temperature for *A. terreus* for lignin peroxidase production (Figure 2a) was 33.6°C (45 U/ml), while that for manganese peroxidase (Figure 2b) was 33.1°C (33 U/ml). Such temperatures lie in the mesophilic range. In this connection Kanayama et al. (2002) found that the optimum temperature of manganese peroxidase production by *A. terreus* strain LD-1 was 37°C. Meanwhile, it was 37 to 40°C for *P. chrysosporium* (Wen et al., 2010).

pH

In this study, the optimum pH for lignin peroxidase production by *A. terreus* (Figure 2c) was 4.1, while maximum yield of manganese peroxidase (Figure 2d) was obtained at pH 5.8. The encountered pH values are in the acidic range. These findings are in accordance with previous literature data. The pH optimum of lignin peroxidase production in *P. chrysosporium* was 5.0 (Alam et al., 2009). The optimum pH for manganese peroxidase

Table 2. Ligninolytic enzymes production (U/ml) of fungal isolates incubated for 10 days at 30°C.

Fungus	Laccase	Manganese peroxidase	Lignin peroxidase
Wadi Degla protectorate			
Alternaria alternata	0.000^{E}	1.6342^{KL}	2.4256^{DEF}
Aspergillus flavus	0.000^{E}	3.3953^{H}	2.4468^{CDE}
Aspergillus fumigatus	0.068^{D}	0.8543^{L}	2.2591^{FG}
Aspergillus ustus	0.000^{E}	6.3084^{F}	2.1673^{G}
Fusarium moniliforum	0.0188^{A}	4.6139^{G}	2.2897^{EFG}
Fusarium pallidoroseum	0.000^{E}	1.4315^{LK}	2.6315^{B}
Penicillium chrysogenum	0.000^{E}	1.9215^{KJI}	3.0757^{A}
Orman garden			
Aspergillus aculeatus	0.000^{E}	9.0508^{D}	2.5283^{BCD}
Aspergillus flavus	0.000^{E}	7.8964^{E}	1.7437^{I}
Aspergillus fumigatus	0.0153^{B}	13.2427^{B}	2.6889^{B}
Aspergillus terreus	0.0157^{B}	20.2194^{A}	3.0198^{A}
Botryotrichum piluliferum	0.000^{E}	1.8625^{IJK}	1.9638^{H}
Cochliobolus lunatus	0.0102^{C}	12.1755^{C}	1.9446^{H}
Emericella nidulans	0.000^{E}	2.2460 ^{IJ}	2.3222 ^{EFG}
Benzene station			
Aspergillus aculeatus	0.000^{E}	5.0218^{G}	1.7437^{I}
Aspergillus flavus	0.000$_{E}$	3.5557^{H}	2.6858^{B}
Aspergillus fumigatus	0.000^{E}	6.5457^{F}	0.9743^{J}
Aspergillus niger	0.000^{E}	1.1762^{KL}	2.6092^{BC}
Aspergillus terreus	0.000^{E}	1.5457	2.6614^{B}
Fusarium pallidoroseum	0.000^{E}	2.2460^{IJ}	0.8874^{J}
Gibberlla accuminata	0.000^{E}	2.4384^{I}	2.4417^{CDE}
LSD (0.01)	0.0019	0.3918	1.1049

The least significant difference (LSD) was calculated at 99% confidence interval (probability of error p=0.01). Means followed by the same letters are statistically non significant.

production by the same organism was 4.5 (Kanayama et al., 2002) and 4.8 (Wen et al., 2010).

Carbon sources

In the current work, Figure 3a shows that D-glucose was the most effective stimulator for lignin peroxidase (100.12 U/ml) as well as manganese peroxidase (199.31 U/ml) production with *A. terreus*. The preference of D-glucose may be due to its ready availability to glycolysis by the fungus.

The present results are in agreement with Hadibarata et al. (2009) who found that glucose led to high lignin peroxidase and manganese peroxidase activities by *Polyporus* spp. S133. Glucose improved lignin peroxidase activity of *Arthromyces ramosus* than other carbon sources (Jing, 2010). Glucose also induced production of manganese peroxidase by *Trametes trogii* CTM 10156 (Levin et al., 2005).

Nitrogen sources

In the present study, the readily available sodium nitrate enhanced the maximum significant yield of lignin peroxidase and manganese peroxidase (Figure 3b). The other tested N-sources caused significant decreases in the studied enzymes. Using the ammonium salts (ammonium chloride and ammonium sulfate) as nitrogen sources caused *A. fumigatus* to achieve maximum activity of lignin peroxidase (Jin et al., 2007). On the other hand, peptone induced highest production of lignin peroxidase and manganese peroxidase by *P. chrysosporium* ME-446 (Kapich et al., 2004) and *Polyporus* spp. S133 (Hadibarata et al., 2009). Meanwhile, best manganese peroxidase production by *Pleurotus pulmonarius* was on ammonium nitrate (Stajic

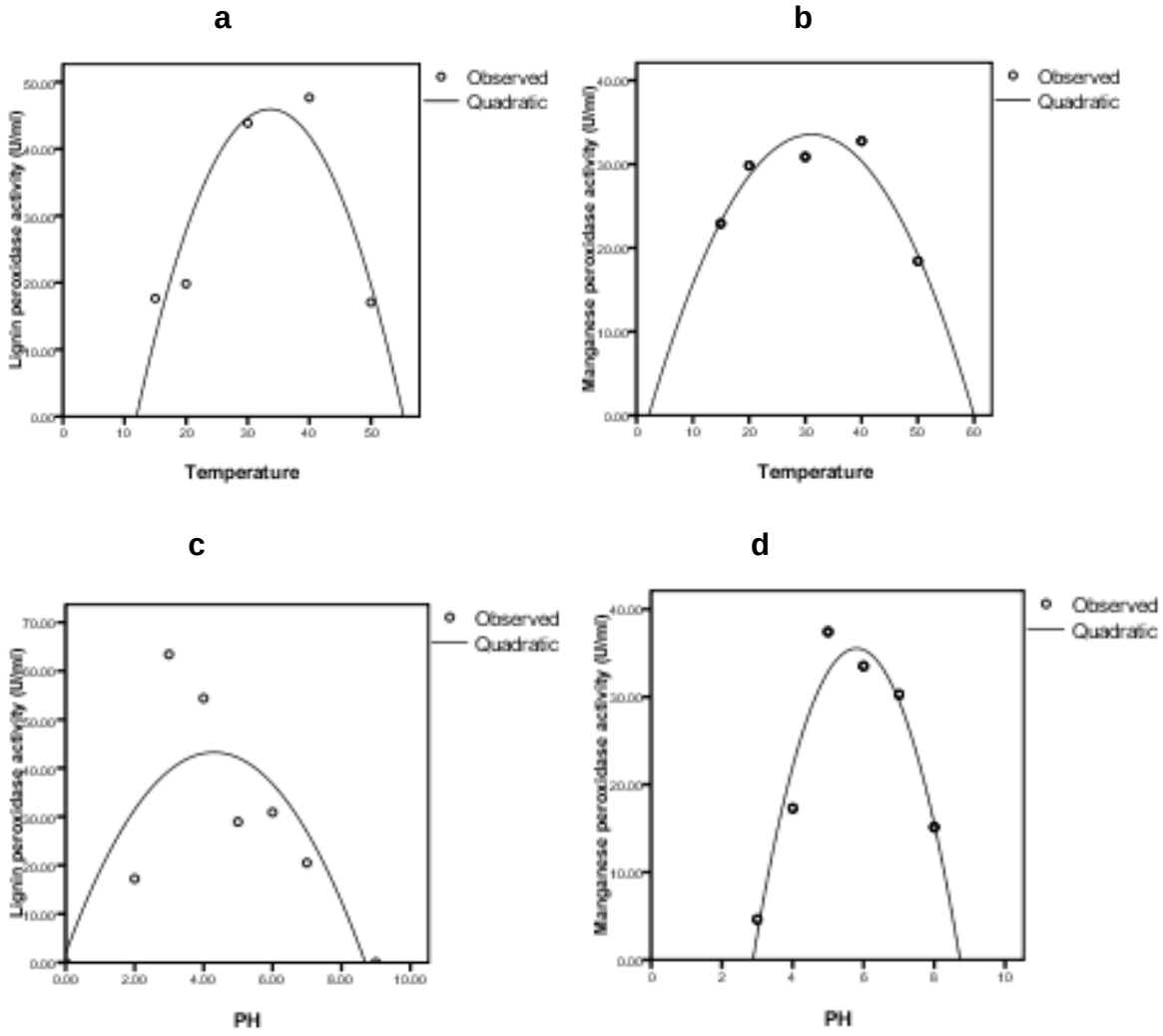

Figure 2. Quadratic regression model fit of ligninolytic enzymes production by *A. terreus*. a: lignin peroxidase production as a function of temperature, R^2=0.854, y= -64.667+ 6.5786x- 0.0978x^2. b: manganese peroxidase production as a function of temperature, R^2=0.899, y= -5.2412+ 2.4945x-0.0401x^2. c: lignin peroxidase production as a function of pH, R^2= 0.676, y= 1.959+ 19.228x - 2.239 x^2. d: manganese peroxidase production as a function of pH, R^2=0.937, y= -104.28+ 48.1981x-4.1539x^2.

et al., 2006).

Biodegradation of PAHs in soil model

Five Gram-sterile soil samples without detectable PAH contamination were spiked with 150 ppm PAH (naphthalene or anthracene) and inoculated with or without *A. terreus* were incubated at room temperature in the dark. Fungal hyphae were observed from the first week on the sterilized soil inoculated with *A. terreus*. After 4 weeks, the soil was completely covered with fungal mycelia. From Figure 4a, it appears that the control (no fungal inoculation) showed a certain degree of naphthalene degradation with time but with a lower rate compared with that observed in *A. terre*us system, which had almost completely degraded (98.5%) naphthalene within four weeks. Figure 4b elucidates the higher rate of *A. terreus* system in degrading anthracene reaching 91% after four weeks compared with no fungal inoculation system. This distinguished degradation ability exceeds that obtained by Acevedo et al. (2011) where *Anthracophyllum discolor* degraded 80% of anthracene after 60 days.

*A. terre*us showed a higher activity (98.5%) in degrading naphthalene which possesses two aromatic rings than anthracene (91.0%) comprising three aromatic rings. These results are in accordance with previous findings (Launen et al., 1995; Leonardi et al., 2007) where microorganisms could degrade more efficiently

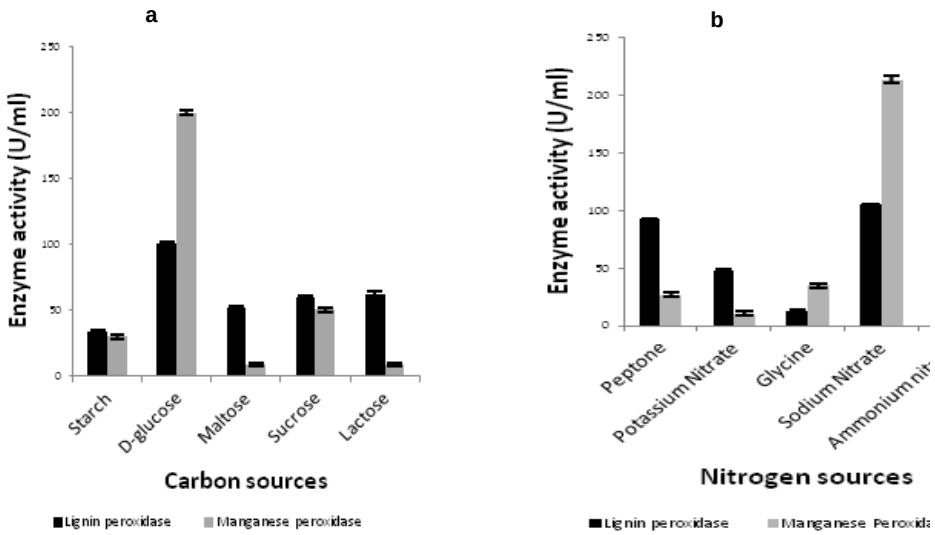

Figure 3. Effect of different used carbon (a) and nitrogen (b) sources on lignin peroxidase and manganese peroxidase production by *A. terreus.* Bars show Means. Error Bars show Mean ± SE.

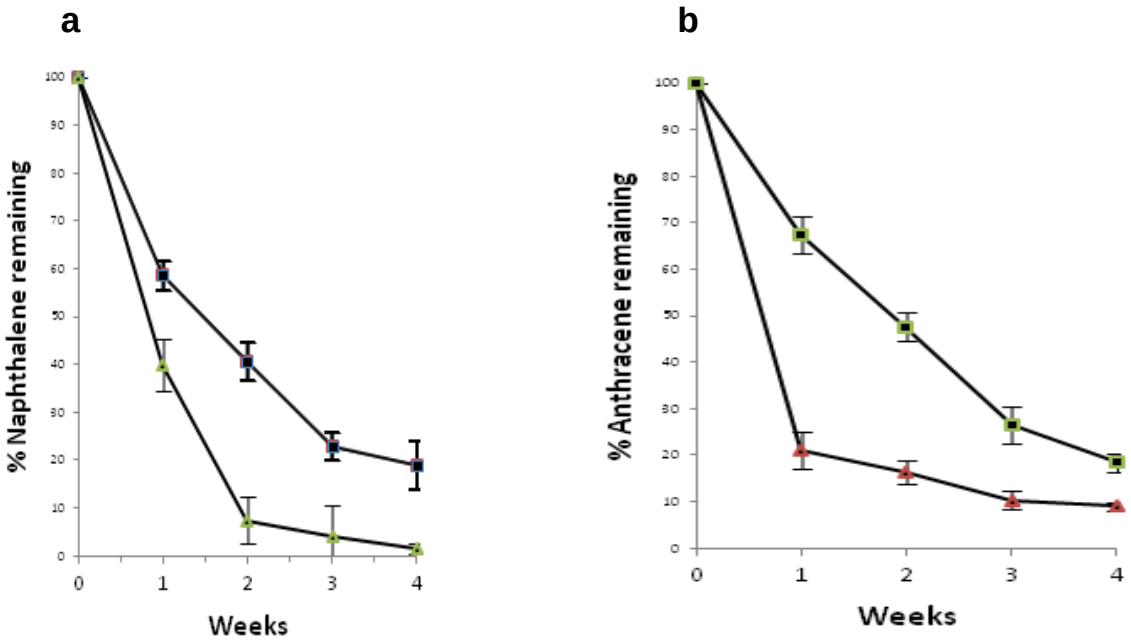

Figure 4. Percentage of PAH degradation by *A. terreus* in soil. a: naphthalene, b: anthracene. Inoculated (▲) and uninoculated control (■) sterilized-soils. Data are mean ± SE.

PAHs with lower number of aromatic rings in the molecule. Contradictly, Silva et al. (2009) found that low molecular weight PAHs (2 to 3 rings) did not appear to be more susceptible to degradation.

From the foregoing discussion, it could be concluded that *A. terreus* isolated from Orman Garden exhibited the highest significant production of manganese peroxidase, lignin peroxidase as well as laccase. The applicable importance of ligninolytic enzymes lies in the fact that they can contribute in breaking down recalcitrant pollutants that may help in reduction of some environmental pollution problems. The saprophytic fungus *A. terreus* from this work showed a remarkable activity in decreasing naphthalene as well as anthracene percentages in soil. It is highly recommended to be used safely in treatment of soils contaminated with PAHs.

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