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# Efficacy of *Trichoderma* fungal species in the removal of α-naphthol from potato dextrose agar media

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## ABSTRACT

Alpha-naphthol is a two-ring aromatic hydrocarbon with toxic and mutagenic properties. Bioremediation technology is considered to be an efficient, economical, and environmentally friendly approach to the remediation of the sites contaminated with polycyclic aromatic hydrocarbons. In this study, six fungal species of the *Trichoderma* genus were cultured in potato dextrose agar (PDA) media containing 10-200 mg/kg of α-naphthol for the adaptation of the fungal strains. The removal of α-naphthol was assessed 30 days after the growth of the adapted fungal colonies at various concentrations of α-naphthol (50, 100, and 150 mg/kg). According to the obtained results, all the fungi could grow in the culture media containing α-naphthol, removing α-naphthol from the media. The highest removal efficiency belonged to *T. viridescens*, while the lowest removal efficiency belonged to *T. koningii.* In addition, the growth ability of the fungi was determined based on the colony diameters, and the results indicated the highest and lowest colony diameters in case of *T. koningii* and *T. viridescens,* respectively. In other words, an inverse correlation was observed between the fungal growth rate and α-naphthol removal efficiency. On the other hand, the results of enzyme activity assay demonstrated that the activity of peroxidase and catalase increased with higher α-naphthol contamination. The highest enzyme activity was observed in *T. viridescens,* growing in the media containing 150 mg/kg of α-naphthol, which indicated a marked correlation between α-naphthol removal efficiency and enzyme activity. Therefore, it could be concluded that *T. viridescens* had the highest enzyme activity and α-naphthol removal efficiency.

**Keywords:** α-Naphthol, Pollution, *Trichoderma*, Bioremediation, Enzymatic activity

## Introduction

The chemicals found in petroleum derivatives are considered to be important chemical pollutants, which are classified into four categories of asphalt, heterocyclic, aliphatic, and aromatic compound.[1, 2] Polycyclic aromatic hydrocarbons (PAHs) are among the most debilitative industrial pollutants, the rate of which has recently increased in air and soil. Air pollutants such as diesel exhaust particles consist of a complex permutation of particulate matter, including elemental carbon and PAHs.[3-5] PAHs are released into the environment through natural and humans processes, such as the burning of fossil fuels and petroleum-derivative fuels.[6] PAHs are important pollutants in the environment due to their toxic, mutagenic, and carcinogenic properties. For instance, phenanthrene could lead to the development of human skin photosensitizers and mild allergy, which denotes its carcinogenic properties.[7-9]

Alpha-naphthol is an aromatic hydrocarbon containing two rings, which is produced during naphthalene hydroxylation in chemical and petrochemical processes. It is a toxic and carcinogenic compound, which could be released into various ecosystems.

Several studies have evaluated the toxic effects of PAHs (e.g., α-naphthol) on the early

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life stages of plants, and the results have indicated that plant growth significantly reduces at the highest tested concentration of this material (375 mg/kg).[6] Furthermore, some studies have investigated the toxic effects of various concentrations of α-naphthol (0.1, 1, 10, 50, and 100 mg/l) on the growth of the roots and hypocotyls of lettuce. Growth inhibition has also been reported in the plants treated by 100 mg/l of benzo[a]pyrene (BaP),[10] while the cytotoxicity of BaP on pollen grains has been observed in *Helianthus annus*.[11] Moreover, the genotoxicity of BaP and the subsequent changes in the DNA content and sequences have been reported in *Trifolium repens* L. and some human cell lines.[12, 13]

Soils contaminated with PAHs, including α-naphthol, are commonly found in gas stations and petroleum, wood impregnation, and industrial sites. Remediation of such sites is of great interest due to the worldwide ubiquitous distribution of these compounds, as well as their low bioavailability and prolonged environmental persistence.[14, 15] White rot fungi, (e.g., *Phanerochaete chrysosporium*) are wood decomposing basidiomycetes, which could degrade not only lignin, but also a broad spectrum of recalcitrant environmental organ pollutants, including PAHs, which are not readily degraded by other microorganisms.[16, 17] This degradative activity occurs due to the lignin-degrading systems in fungi and is associated with enzymes such as lignin peroxidase, manganese peroxidase, and laccase.[18, 19]

Few studies have been focused on the bioremediation of PAHs using other fungal strains.[17, 20] The present study aimed to assess some of the *Trichoderma* fungal strains that commonly grow in agricultural soils (e.g., wood digesting fungi) and their efficiency in the removal of α-naphthol from contaminated media.

## Materials and Methods

### *Fungi collection*

In this study, six fungal strains were obtained from the mycology laboratory of Bu-Ali Sina University in Hamadan, Iran, including *T. viridescens* (ACTT 28038), *T. orientals* (locally isolated), *T. atroviride* (ATCC 20476), *T. asperellum* (ATCC 52438), *T. harzianum* (locally isolated), and *T. koningii* (ATCC 26113). The fungi were cultured on potato dextrose agar media (PDA; Merck, Darmstadt, Germany) for renewal. The culture media contained lactic acid (1.5 ppm) and rose bengal (0.04 mg/l) for bacterial growth inhibition. The samples incubated at the temperature of 25±2 °C for four days (device obtained from Pars Co., Tehran, Iran).

### *Adaptation of fungi to α-naphthol*

The fungal species were adapted for growth in the media containing α-naphthol. To do so, the samples were cultured on the media containing 10 mg/kg of α-naphthol, and one colony was injected into each petri. In this study, α-naphthol was purchased from Sigma-Aldrich Company (USA).

The growing colonies were cultured on the PDA media containing 20 mg/kg of α-naphthol. Through a similar process, the fungi were cultured on the media with increased concentrations of α-naphthol (10-200 mg/kg). The step-by-step increasing of the α-naphthol concentration provided the opportunity for the adaptation of the fungi against α-naphthol contamination. Following that, the growing colonies were collected for further experimentation. In addition, a growth assay was conducted through the comparison of the colony diameters of the fungal strains growing in the media contaminated with α-naphthol at the concentrations of 50, 100, 150, and 200 mg/kg. The colony diameters of the fungal mycelia were measured using a measuring tape and compared with the control plates.

### *Removal of α-naphthol*

After purification and adaptation, all the isolated fungal strains were cultured on PDA containing 50, 100, and 150 mg/kg of α-naphthol and incubated at the temperature of 25±2 °C for 45 days. In the control (blank) plates, PDA was combined with α-naphthol and preserved in the same conditions without the fungal colonies. The plates were subjected to the same procedures and incubated in the same conditions as the experimental plates.

In order to measure the remaining α-naphthol on the PDA media after 30 days, the contents of the plates were homogenized, and two grams of the contents of each plate were mixed with five milliliters of hexane as the solvent and shaken for three minutes. After the separation of the organic phase from the aqueous phase, the organic phase was collected and preserved in a microtube for the evaporation of the solvent. Following that, the solution was filtered using a Millipore (0.22 µ) and stored in a microtube for gas chromatography analysis.

***Analysis of α-naphthol***

At this stage, 100 microliters of the prepared was subjected to gas chromatography, and the concentrations of α-naphthol on the PDA media were measured using the Varian Star 3400 Gas Chromatograph, equipped with a flame ionization detector and a BPX-5 capillary column (25x0.22 mm), which operated as described previously.[21] The peak areas of the internal standard and α-naphthol were used to calculate the peak area ratios. The ratios of the experimental and control samples were compared to the α-naphthol standards.

***Enzyme assay***

In order to prepare the extraction buffer, Tris (1.2 g), ascorbic acid (0.1 g), sucrose (17.2 g), cysteine chloride (0.1 g), and hydrochloric acid (26.8 ml, 0.2 normal) were mixed and reached 100 milliliters using distilled water (pH=7.5). Afterwards, one gram of each fungal mycelium was combined with five milliliters of the extraction buffer and homogenized for 30 minutes. The extraction procedure was performed at the temperature of -4 °C.

Following that, the samples were centrifuged in the refrigerated centrifuge (Eppendorf, 5417R, Germany) at 12,000 rpm and temperature of 4 °C for 15 minutes, and the supernatant solution was stored at the temperature of -20 °C. In order to determine the peroxidase activity, 0.1 milliliter of the prepared extract was poured into the test tube, and two milliliters of buffered acetate (0.02 M; pH= 4.8), 0.2 milliliter of 3% hydrogen peroxide, and 0.2 milliliter of benzidine (0.02 M in 50% methanol) were added. Afterwards, the light absorption of the solution was evaluated at the wavelength of 530 nanometers using a spectrophotometer (Biowave II, England). Finally, enzyme activity was defined as the absorption unit per minute for each milligram of protein.[22]

Catalase activity and $H_2O_2$ reduction were evaluated based on the absorbance changes at 530 nanometers. To this end, 0.2 milliliter of the extract was poured into the test tube, and 2.5 milliliters of phosphate-buffered saline (pH=4.8) and 0.2 milliliter of 3% hydrogen peroxide were added. Changes in the absorbance were measured at the wavelength of 530 nanometers, and enzyme activity was defined as the absorption unit per minute for each milligram of protein.[19]

***Statistical analysis***

The experimental results were statistically processed by calculating the mean values using Duncan's test at the levels of 1% ($P \leq 0.01$) and 5% ($P \leq 0.05$) based on a completely randomized design. Data analysis was performed in SAS version 9.1, and the data were expressed as the mean and standard error (SE) of a minimum of 3-5 samples in the experimental and control groups.[23]

## Results and Discussion

***Fungi adaptation to α-naphthol***

The fungi were cultured on the media containing increasing concentrations of α-naphthol (10-200 mg/kg) 10 mg/kg at a time. This procedure induced resistance in the fungi against α-naphthol contamination. The growth activity of the six fungal strains was measured at various concentrations of α-naphthol (50, 100, 150, and 200 mg/kg) and expressed as the diameters of the fungal colonies (Table 1). According to the findings, all the fungi were adapted and resistant to the mentioned concentrations of α-naphthol. In addition, they produced adequate colonies at the α-naphthol concentration of 50 mg/kg (diameter: 100 mm), while only some fungi maintained their growth activity at up to 150 mg/kg of α-naphthol.

The obtained results indicated that

*T. orientale* had the highest growth (colony diameter: 85 mm), whereas *T. viridescens* had the lowest growth (colony diameter: 30 mm) at the α-naphthol concentration of 150 mg/kg. At the α-naphthol concentration of 200 mg/kg, the fungal growth was observed to relatively decrease, with the lowest growth detected in *T. viridescens* (diameter: 10 mm), and the highest growth observed in *T. koningii* (diameter: 65 mm).

According to the results of the present study, the fungi could grow in the media containing various concentrations of α-naphthol (10-200 mg/kg). In other words, α-naphthol contamination could not inhibit the growth of the fungal strains significantly, while exerting no evident toxic and detrimental effects on the fungal strains. Therefore, it could be inferred that the fungi were resistant to the toxic effects of α-naphthol at the applied concentrations, which is in line with the previous findings on BaP.[14, 15, 24] This might be due to the fact that fungi use α-naphthol as a nutrient for survival, growth, and metabolism using extracellular enzymes to break down the recalcitrant hydrocarbon molecules, thereby converting α-naphthol into simpler products that could be absorbed for their growth and nutrition; this finding is also consistent with the previous studies in this regard.[15, 18, 25, 26] Several studies have isolated some bacteria and fungi from petroleum-contaminated soils.[27- 30]

Table 1. Growth assessment of studied fungi in media containing various concentrations of α-naphthol based on colony diameter (mm)

| Fungal strains/ Concentrations | | α-Naphthol (mg/kg) | | | |
|---|---|---|---|---|---|
| | | 50 | 100 | 150 | 200 |
| *T. asperellum* | ATCC 52438 | 100 | 79 | 70 | 55 |
| *T. atroviride* | ATCC 20476 | 100 | 90 | 80 | 60 |
| *T. harzianum* | Locally isolated | 100 | 70 | 65 | 30 |
| *T. viridescens* | ACTT 28038 | 100 | 45 | 30 | 10 |
| *T. orientals* | Locally isolated | 100 | 90 | 85 | 60 |
| *T. koningii* | ATCC 26113 | 100 | 82 | 67 | 65 |

### *Removal of α-naphthol*

Soil pollution with various chemicals (e.g., petroleum and its derivatives) cause an environmental disaster. Bioremediation technologies involve the use of plants and microorganisms to reduce, eliminate or transform the contaminants found in soil, sediments, and water. Microorganisms, especially fungal strains, play a pivotal role in the decontamination of contaminated media.[24, 25]

In the current research, six fungal species (*T. viridescens, T. orientale, T. atroviride, T. asperellum, T. harzianum,* and *T. koningii*) were cultured on the PDA media containing 50, 100, and 150 mg/kg of α-naphthol. After 30 days of fungal growth in the media containing various concentrations of α-naphthol, residual α-naphthol was extracted and measured in percentages. With the initial percentage of α-naphthol that was added to each plate, the removed α-naphthol level was calculated in the experimental and control plates. The obtained results indicated that the samples fungi could significantly remove α-naphthol from the PDA media.

At the α-naphthol concentration of 50 mg/kg in the control plates, the contamination level of α-naphthol was observed to decrease (12%), which was attributed to evaporation (non-biological removal). Furthermore, the findings of the current research indicated that in the presence of the fungal strains, α-naphthol removal increased significantly. The lowest rate of α-naphthol removal belonged to *T. koningii* (51%; evaporation included), while the highest rate was observed with *T. viridescens* (81%; evaporation included). Therefore, it could be concluded that all the tested fungi were able to remove α-naphthol from the media at the concentration of 50 mg/kg (Fig. 1).

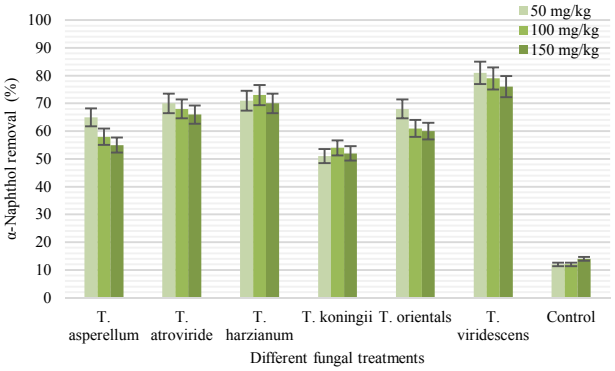


Fig. 1. Removal of α-Naphthol (%; evaporation included) by studied fungal strains growing at α-naphthol concentrations of 50, 100, and 150 mg/kg (All fungal strains significantly decreased α-naphthol in media; P≤0.01)

In the control plates, the reduction of α-naphthol at the concentration of 100 mg/kg was estimated at 12%. In the experimental groups,

the lowest rate of α-naphthol removal was observed with *T. koningii* (54%; evaporation included), while the highest rate was detected with *T. viridescens* (79%; evaporation included). Moreover, the obtained results indicated that all the studied fungi were able to remove α-naphthol from the media containing 100 mg/kg of α-naphthol (Fig. 1).

At the α-naphthol concentration of 150 mg/kg, *T. koningii* (52%; evaporation included) and *T. viridescens* (76%; evaporation included) resulted in the lowest and highest removal rate of α-naphthol, respectively. On the other hand, the findings of the current research demonstrated that the all fungi were able to remove α-naphthol from the PDA media containing 150 mg/kg of α-naphthol (Fig. 1).

According to the results of the present study, all the fungal strains were able to remove α-naphthol from the media containing 50-150 mg/kg of α-naphthol. Some of the reports in this regard have suggested that the media containing 50 mg/kg of α-naphthol represents the maximum value that is tolerated by most fungi, while our findings indicated that the fungi could grow and remove α-naphthol from the PDA media containing 50-150 mg/kg of α-naphthol. Therefore, it could be inferred that the studied fungi were able to remove α-naphthol, showing acceptable performance at higher concentrations of α-naphthol.[17] Furthermore, the results of the present study demonstrated that all the fungi were effective in the removal of α-naphthol from the media at most the used concentrations, which makes them an optimal candidate for the elimination of α-naphthol contamination. Our findings in this regard are consistent with the results of some studies investigating PAHs.[14, 17, 24, 29, 31, 32] On the other hand, the results of the present study indicated that *T. viridescens* had the highest efficiency in the removal of α-naphthol.

According to the findings of the current research, the removal efficiency of α-naphthol could be enhanced in the presence of fungi, which is in line with some of the previous studies in this regard.[16, 21, 25, 33] For instance, Romero *et al.* could degrade PAHs containing BaP using soil filamentous fungi.[34] Other researchers have also performed the degradation of PAHs (2-7 rings) in microaerobic and very-low-oxygen conditions using soil fungi.[35] Although several reports have been published regarding the function of some bacteria in the removal of α-naphthol, this is the first report on the bioremediation ability of *Trichoderma* fungal species against α-naphthol contamination.[36, 37]

***Enzyme activity***

Fig. 2 depicts the results of catalase activity assay at various concentrations of α-naphthol (50, 100, and 150 mg/kg). As can be seen, there was significant correlation between the increased enzyme activity and higher α-naphthol concentration (50 and 100 mg/kg). In addition, the enzyme activity of the fungi increased significantly in the group treated with α-naphthol ($P \leq 0.01$). The minimum enzyme activity was observed in the control group, and the maximum activity was denoted in the group treated with 100 mg/kg of α-naphthol (Fig. 2).

According to the findings of the current research, catalase activity decreased in the group treated with 150 mg/kg of α-naphthol in some cases. For instance, the measurement of catalase activity in *T. viridescens* indicated that the control group had the lowest enzyme activity ($3\times10^{-3}$ unit/mg protein), while the highest activity was observed in the fungi growing at the α-naphthol concentration of 100 mg/kg ($1.8\times10^{-2}$ unit/mg protein). On the other hand, the enzyme activity decreased to $8\times10^{-3}$ unit/mg protein in the group exposed to 150 mg/kg of α-naphthol.

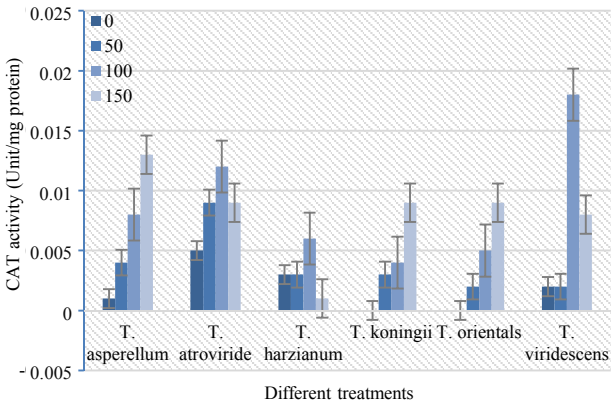


Fig. 2. Catalase activity (unit/mg protein) in fungal strains exposed to various concentrations of α-naphthol (Catalase activity increased at higher α-naphthol concentrations; significant differences between various treatments; $P \leq 0.01$)

Fig. 3 shows the peroxidase activity in the fungal strains. As is observed, peroxidase activity increased at the higher concentrations of α-naphthol. However, enzyme activity had a

significant difference in the groups treated with various concentrations of α-naphthol (P≤0.01), with the highest peroxidase activity observed in the groups treated with 150 mg/kg of α-naphthol, and the lowest activity denoted in the control plates (no α-naphthol treatment). For instance, the lowest ($0.5\times10^{-3}$ unit/mg protein) and highest peroxidase activity for *T. viridescens* ($9\times10^{-3}$ unit/mg protein) was determined in control samples and the group treated with 150 mg/kg of α-naphthol, respectively. In some of the fungal strains (*T. atroviride* and *T. orientale*), peroxidase activity increased at the α-naphthol concentrations of up to 100 mg/kg, while it decreased in the group treated with 150 mg/kg of α-naphthol. It is notable that the activity of the other enzymes also increased in the group treated with 150 mg/kg of α-naphthol.

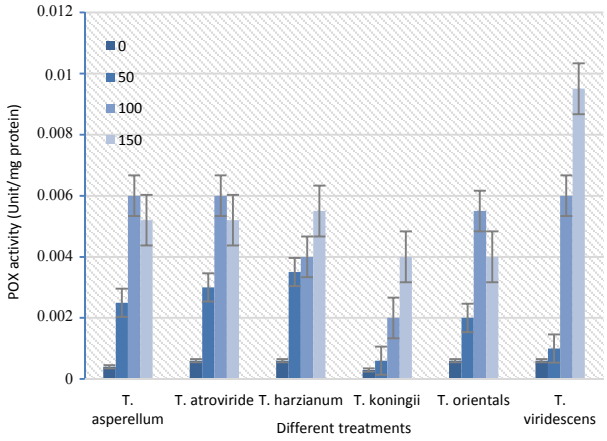


Fig. 3. Peroxidase activity (unit/mg protein) of studied fungal strains at various concentrations of α-naphthol (Peroxidase activity increased at higher α-naphthol concentrations; significant differences between various treatments; P≤0.01)

The results of the enzyme assay in the present study demonstrated that the activity of various enzymes was significantly affected by various concentrations of α-naphthol. Furthermore, the enzyme assay of the studied fungi indicated that peroxidase and catalase activity increased at the higher concentrations of α-naphthol. In this regard, the lowest and highest peroxidase activity was observed with *T. koningii* and *T. viridescens*, respectively, while the highest and lowest catalase activity was denoted in *T. viridescens* and *T. harzianum*, respectively. The findings of the current research revealed a significant correlation between the enzyme activity of the fungi and their ability to remove α-naphthol. For instance, the enzyme activity was observed to decrease in the group treated with 150 mg/kg of α-naphthol, and the reduction was attributed to *T. koningii* and *T. orientale* in case of peroxidase, while it was attributed to *T. atroviride* and *T. harzianum* in case of catalase. Therefore, it could be concluded that 150 mg/kg of α-naphthol caused toxicity in the enzyme activity of the fungi.

According to the results of the present study, *T. viridescens* had the highest enzyme activity and efficiency in the removal of α-naphthol. Few reports in this regard have also confirmed the high enzyme activity of the soil contaminated with petroleum derivatives.[38-40] Moreover, some studies have denoted that microorganisms such as *Phanerochaete chrysosporium* and *Aspergillus niger* are capable of producing enzymes that could be effectively utilized as biodegradation agents in waste recycling processes and bioremediation of petroleum-contaminated sites.[41-43]

## Conclusion

According to the results, α-naphthol could not inhibit the growth and multiplication of microorganisms such as *Trichoderma* species as their growth even increased in the media contaminated with α-naphthol. In other words, these fungal species were able to use α-naphthol as a nutrient source. Our findings also demonstrated that the fungal species were effective in the removal of α-naphthol from the PDA media, and the highest removal efficiency was observed in *T. viridescens*. Therefore, the use of this species is recommended for the bioremediation of the soil and media contaminated with α-naphthol. Our bibliographical studies indicated that this was the first report regarding the bioremediation potency of *Trichoderma* species.

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