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NAPHTHALENE AND PHENANTHRENE BIODEGRADATION BY ANTARCTIC SOIL - ISOLATED *ASPERGILLUS FUMIGATUS* STRAINS

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ABSTRACT

This article describes the experiments with the two investigated fungal strains isolated from soils on Livingston Island, Antarctica. Our studies by gas chromatography - mass spectrometry (GC - MS) analyses showed the ability degree of the strains to degrade low molecular weight polyaromatic compounds such as naphthalene and phenanthrene. The different degradation capacity of both strains towards each one of the compounds studied was established. The amount of naphthalene in the medium decreased by 44 % during the 9 days cultivation of Aspergillus fumigatus AL3, while it decreased by 37 % when grown in a medium inoculated with Aspergillus fumigatus AL9. The strain A. fumigatus AL3 was able to reduce the phenanthrene amount in the medium by 44.5 %, whereas A. fumigatus AL9 reduced it by a significant 90.5 % under the same conditions and the same period. Some of the intermediates, such as naphthalene - 1, 2 - diol, 2 - hydroxybenzaldehyde, 2 - hydroxybenzoic acid, naphthalene - 1 - ol, benzene - 1, 2 - dicarboxylic acid, and benzene - 1, 2 - diol, in the catabolite chain of both compounds were also identified. They are typical for the biodegradation of the investigated compounds also with the help of other types of microorganisms.

Keywords: naphthalene, phenanthrene, degradation, intermediates, Aspergillus fumigatus.

INTRODUCTION

The development of modern technologies is increasingly aligned with the need to protect the purity of the environment. However, the long period of industrialization has been associated with production processes that have generated waste and pollution from various chemical compounds harmful to nature and human health [1]. One of the most widespread, toxic and difficult to degrade of these is the well-known polyaromatic hydrocarbons (PAHs).

Low - molecular - weight polyaromatic compounds are typically used in the production of certain polymers, explosives, insecticides, and pharmaceuticals [2, 3]. Active volcanoes, naturally occurring forest fires, bitumen and asphalt production facilities, coal, coke, and oil extraction operations, and petroleum refineries are the most well - known natural and industrial sources of PAHs. Contemporary analytical methods like gas chromatography combined with mass spectrometry improve the ability to accurately measure PAHs in the environment, food, and other materials [4, 5].

The U. S. EPA has set a reference concentration (RfC) for non-cancer effects 0.003 g L^{-1} for naphthalene. Additionally, the EPA has a Lifetime Health Advisory (LHA) of 0.0001 g L^{-1} (100 ppb) and a drinking water equivalent guideline (DWEL) of 0.0007 g L^{-1}. The ambient water quality limit for phenanthrene, as recommended by the U.S. EPA is 0.00005 g L^{-1}. Numerous considerations, such as the possibility of carcinogenicity and other detrimental health effects, form the basis of these recommendations.

Much effort has been made to create ways for their removal from contaminated soils and waters. It is believed that remediation using biological methods ranks among the most environmentally friendly approaches, as it allows for the degradation of pollutants without producing other toxic compounds as intermediate or final products in the ongoing biochemical reactions [6 - 8]. It can be argued that this is also an economically viable method. Representatives from different types of microorganisms significantly contribute to bioremediation.

Although numerous reviews and research publications have been authored on the capability of bacteria and fungi to degrade and use both low - molecular and high - molecular PAH as carbon and nitrogen sources, research activities continue [9 - 14]. With the advancement of knowledge and research in microbial diversity, an increasing number of new microbial strains capable of removing PAH and their use in developing biotechnologies for environmental cleaning are being discovered.

The exceptional ability of fungi to metabolize a variety of substrates is due to the number of specific enzymes they possess. When studying the degradation of aromatic and polyaromatic xenobiotics, a large share is devoted to the action of their lignolytic enzymes [15, 16]. However, there are other possibilities for the degradation and absorption of some of these compounds. The presence of catechol dioxygenase, hydroquinone oxygenase, and other enzymatic activities suggests the presence of intracellular mechanisms for the metabolism of mono - and polyphenolic compounds [17, 18].

Microbial strains possessing similar properties are discovered in various and diverse geographical regions. Of particular interest are those located in habitats with extreme living conditions, such as the isolated Antarctic soils and waters [19 - 21]. Their presence there can be explained by the dispersal of spores through air currents, the distribution of pollutants in the oceans due to petroleum spills, decaying wood, and other factors that have accumulated over time.

In the scientific literature, there are already established genera of fungi with significant degradation activity towards various difficult to degrade chemical substrates, including polyaromatic compounds. Such is the genus Aspergillus, whose representatives successfully degrade and utilize several compounds such as: monoaromatic and polyaromatic compounds, microplastics, azo dyes, crude oil and others [22 - 28]. The biochemical investigation of degradation capacity against some aromatics in the most active strains is in progress.

In our previous work, we have shown the ability of two Antarctic *A. fumigatus* strains to degrade and assimilate monoaromatic compounds during their growth [29 - 31]. That indicates a remarkable potential for bioremediation applications in contaminated environments.

The purpose of the present investigation was to discover the metabolic capabilities of the investigated *A. fumigatus* strains concerning naphthalene, anthracene, and phenanthrene, which were applied as the sole carbon and energy sources in the culture medium.

EXPERIMENTAL

Chemicals

The specified later Czapek Dox medium components, along with naphthalene (99.0 %), anthracene (99.0 %), and phenanthrene (97.0 %), were all obtained from Sigma-Aldrich, USA.

Conditions of culture and microorganisms

On Livingston Island in West Antarctica, close to Bulgarian Antarctic base “St. Kliment Ohridski” a permanent, soil samples were taken.

A liquid Czapek Dox medium augmented with 1 % glucose and contained 2 g L^{-1} $NaNO_3$, 1 g L^{-1} KH_2PO_4, 0.5 g L^{-1} KCl, 0.5 g L^{-1} $MgSO_4 \times 7 H_2O$, and 0.01 g L^{-1} $FeSO_4 \times 7 H_2O$ was initially used to cultivate the strains *A. fumigatus* AL3 and AL9 [33]. The medium’s pH was brought to 5.5 by adding a dissolved NaOH.

The fungal pellets that had grown in the liquid nutrient media were leached with sterile distilled water to remove any leftover glucose. 0.07 g of mycelium (wet weight) was used for inoculation of 0.02 L of Czapek Dox medium containing 0.3 g L^{-1} naphthalene, anthracene, or phenanthrene as the only carbon origin. The used flasks (0.3 L) were shaken at 320 rpm in the dark at 23°C using a rotary shaker (IKA KS 130 Basic, GmbH&Ko.KG, Germany). Inoculated flasks served as a control at each stage. The Yeast Extract/ Peptone medium (10.0 g L^{-1}/ 20.0 g L^{-1}) was utilized to follow strain's development in the presence of organic components without tested toxic substances.

PAH extraction and GC-MS analysis

The PAHs were extracted from 0.002 L solution aliquots using 0.002 L of dichloromethane (DCM) three times in an ultrasonic cleaner B-3001 (VWR Int., Belgium). The mixed dichloromethane extracts were concentrated using a rotary vacuum concentrator RVC 2-25 CD plus (Christ, Osterode am Harz, Germany) before GC-MS analysis.

Using a Hewlett Packard 7890 appliance coupled to MSD 5975 equipment (Hewlett Packard, Palo Alto, CA, USA), the GC-MS analysis was performed in EI mode at 70 eV. The HP-5 MS column that was used has dimensions of 30 m x 0.0025 m x 0.00000025 m.

Helium was used as a carrier gas at a flow rate of 1.0 mL min^{-1}. The applied temperature mode was increased by 25°C min^{-1} to 195°C after 24 s at 50°C, and it was kept there for 1.5 min. Following then, the temperature increased to 265°C at a rate of 8°C $min^{-1,}$ then to 315°C at a rate of 20°C min^{-1}, where it stayed for 1.25 min. The ion source temperature was 250°C, while the interface temperature was 280°C [34].

RESULTS AND DISCUSSION

Degradation of PAHs

Most biodegradation investigations on LMW PAHs have been undertaken by researchers on Basidiomycota, particularly lignolytic fungi [35]. Nonetheless, there is some proof that Ascomycota members may as well degrade hazardous chemical substrates such phenolic chemicals, plastics, low - molecular - weight PAHs, and others. The strains of Aspergillus, Penicillium, Trichoderma, and Fusarium have been proven as degraders of organic environmental pollutants [17, 30, 36, 37].

According to 18S rDNA analysis, the isolates of *A. fumigatus* AL3 and AL9 under study are taxonomically related and have been assigned accession numbers by the NCBI, correspondingly Acc. N KT781127.1., and Acc. N JQ639072.1. It has previously been reported that the strain has a good ability for biodegradation of benzenol and other monophenolic compounds, including benzene - 1, 2 - diol, benzene - 1, 4 - diol, 2 - methylphenol, 3 - methylphenol, and 4 - methylphenol [30, 31].

First, the strains' capacity to use naphthalene, anthracene, or phenanthrene in a solid mineral medium (CzapekDox) was examined. In the media containing 0.3 g L^{-1} of naphthalene and phenanthrene, the strains developed in a moderate manner. The strains' development in the mineral medium, which contained 0.3 g L^{-1} anthracene, was substantially slower. One reason for the lack of positive results may be the extremely low solubility of anthracene in water - 0.04 g L^{-1} at 25°C. Of the three compounds, naphthalene has the highest solubility in water - 31 g L^{-1} at 25°C. Phenanthrene has a water solubility of 1.1 g L^{-1} at 25°C [38].

Additional cultivation and degradation tests in a carbonless liquid Czapek Dox medium was conducted, supplemented with 0.3 g L^{-1} of each of the three PAHs under investigation as the only carbon sources. GC - MS studies were performed after the supposedly decreased concentrations of the used polyaromatic chemicals included in the medium decreased. It is evident by tracking the compounds' degradation over a period of nine days that both strains digest the chemicals in different amounts while maintaining the same culture conditions (Figs. 1, 2).

Naphthalene was reduced more quickly by strain *A. fumigatus* AL3 than by strain *A. fumigatus* AL9. The amount of naphthalene in the media decreased by 44 % during the 9 days cultivation of *A. fumigatus* AL3, while it decreased by 37 % when grown in a medium inoculated with *A. fumigatus* AL9.

In the phenanthrene experiments, the strain *A. fumigatus* AL3 can reduce the amount of phenanthrene in the medium by 44.5 % after the concentration decreases up to the ninth day of cultivation, whereas *A. fumigatus* AL9 reduces it over 90.5 % under the same conditions and for the same amount of time (Fig. 3).

The illustration indicates that both strains possess comparable capabilities for naphthalene degradation. However, with respect to phenanthrene, a difference of more than two times in favour of *A. fumigatus* AL9 was observed. Our data demonstrated that the strains' efficacy in degrading both chemicals differed.

It is reasonable to think that phenanthrene would degrade more slowly than naphthalene because of its angular structure, which is thermodynamically more stable. Nonetheless, other researchers consider that the

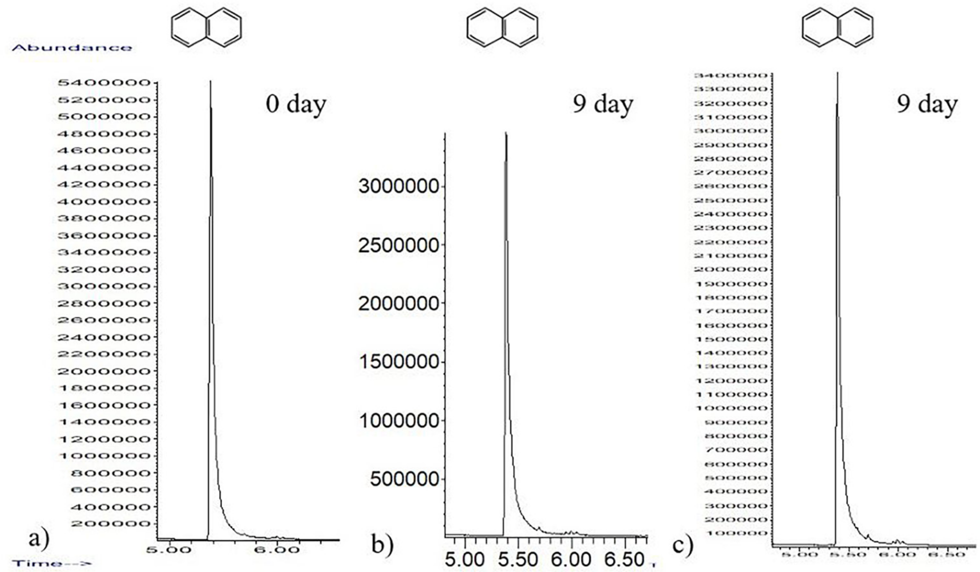

Fig. 1. Gas chromatographic analysis of the reduction in the naphthalene concentration during *Aspergillus fumigatus* strains AL3 and AL9 culturing: (a) naphthalene - initial concentration (0.3122 g L^{-1}); (b) naphthalene after 9 days cultivation with AL3 (0.1748 g L^{-1}); (c) naphthalene after 9 days cultivation with AL9 (0.1969 g L^{-1}).

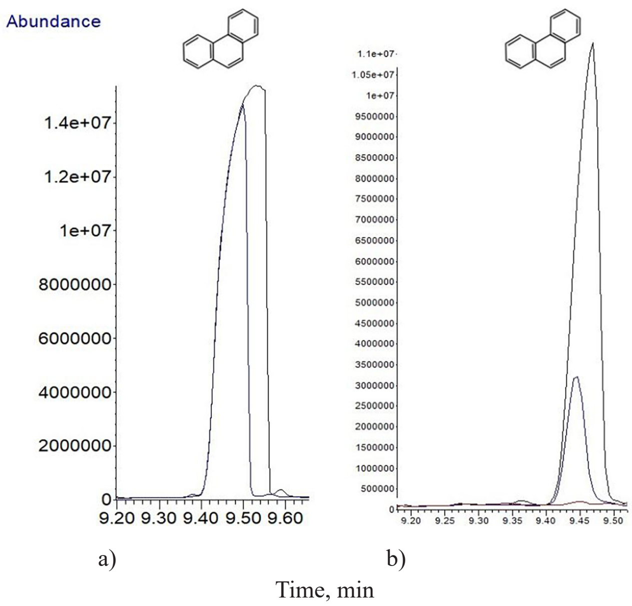

Fig. 2. Gas chromatographic analysis of the reduction in the phenanthrene concentration during *Aspergillus fumigatus* strains AL3 and AL9 culturing: (a) phenanthrene - initial concentration (0.3016 g L^{-1}) and a concentration after 9 days of AL3 cultivation (0.1675 g L^{-1}); (b) phenanthrene - initial concentration (0.316 g L^{-1}) and a concentration after 9 days of AL9 cultivation (0.0295 g L^{-1}).

exposed region, referred to as the bay region between the angled benzene rings, can be targeted by the enzymes more easily than the linear or clustered ones [39]. A reason for this significant difference is most likely due as well to different levels of key enzymatic activities directly related to the process of utilization of naphthalene and phenanthrene in the different strains. The catechol 1, 2 - dioxygenase activity in *A. fumigatus* AL9 cells grown in a medium containing benzenol or benzene - 1, 2 - diol (0.427 - 0.432 U mg^{-1} protein) as the only carbon sources is known to be much higher than that of *A. fumigatus* AL3 cells (0.189 - 0.276 U mg^{-1} protein), according to our earlier research [30, 31]. The metabolism of aromatic compounds is known to be significantly influenced by the enzyme 1, 2 - catechol dioxygenase [40]. Its catalytic activity determines the efficient degradation of complex PAHs to non - toxic compounds that can be incorporated into the tricarboxylic acid cycle [41]. Dioxygenases cleave the several catechol derivatives that are often produced during the PAH degradation.

Catabolic intermediate compounds

When monitoring the reduction of naphthalene and phenanthrene by GC - MS analyses, the emergence of

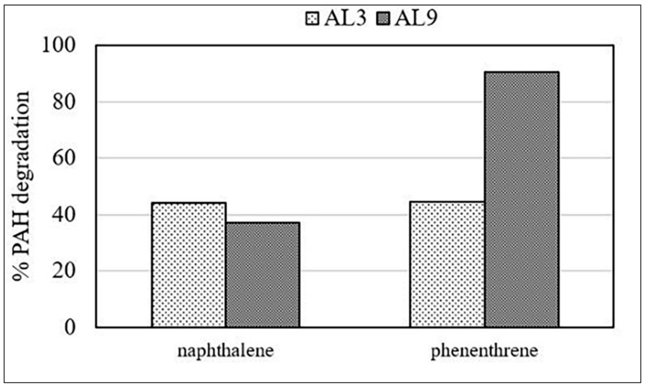

Fig. 3. Comparison of the degree of degradation of naphthalene and phenanthrene, included in a mineral nutrient medium Czapek Dox as sole carbon sources, by *Aspergillus fumigatus* strains - AL3 and AL9.

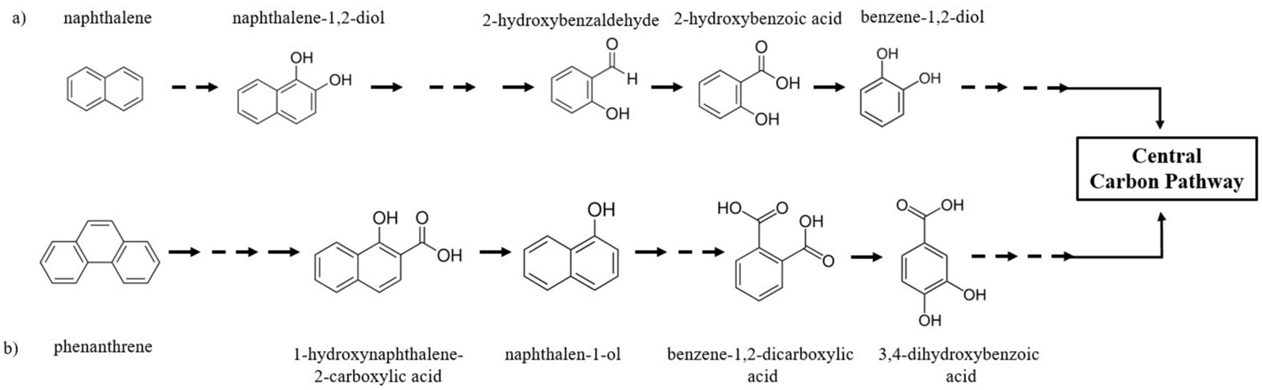

Fig. 4. The intermediate compounds obtained in the catabolic pathways of (a) naphthalene and (b) phenanthrene degraded by *Aspergillus fumigatus* strains - AL3 and AL9.

intermediate compounds was observed. Several of them were recognized. The compounds that are identified in their known chains of degradation, which lead to compounds that are involved in the regular metabolism of substances in microbial cells, are shown in Fig. 4a, b. The described intermediates were found in both studied strains - AL3 and AL9.

They appeared on the 9th day of cultivation and subsequently disappeared, probably due to their rapid transformation and utilization in the degradation process. The intermediates frequently persist in very small concentrations and for very brief periods of time, so it is challenging to monitor them all.

The observed intermediates are typical for other published in the scientific literature schemes of microbial degradation of naphthalene and phenanthrene [40, 42].

2 - hydroxybenzaldehyde and naphthalene - 1, 2 - diol have also been shown to be involved in *Pseudomonas putida* CSV86's degradation of naphthalene [43]. Compounds such as 2- hydroxybenzoic acid, and benzene - 1, 2 - diol are present in practically every degradation process of aromatics, hence they cannot be used as precise indicators of a particular biochemical catabolic pathway.

The discovered compound 1 - hydroxynaphthalene - 2-carboxylic acid during the degradation of phenanthrene, as well as naphthalen-1-ol, benzene - 1, 2 - dicarboxylic acid, and 3, 4 - dihydroxybenzoic acid, give reason to assume that in these molds, phenanthrene has degraded via the phthalic acid pathway described in the facultative or obligate aerobic bacteria Aeromonas, Alcaligenes, Bacillus, and Micrococcus [44, 45]. The same compounds were obtained in similar experiments with *Aspergillus glaucus* strain AL1 [36].

CONCLUSIONS

The results presented in this manuscript, obtained in experiments provided with the *A. fumigatus* strains, add to the limited database about the degradation abilities of ascomycetous fungi to degrade and utilize hazardous chemical substrates.

Our observations indicated that the strains of *A. fumigatus* we studied exhibited varying effectiveness in decomposing both naphthalene and phenanthrene. The two fungal strains were able to degrade and utilize w0.3 g L^{-1} of investigated compounds to different degrees. The strain *A. fumigatus* AL9 was very active toward phenanthrene. It is important to note that according to the U.S. Environmental Protection Agency (EPA), the permissible concentrations of the PAHs we studied are much lower than their concentration applied in the experiments described in the article.

The intermediates identified in the GC - MS analysis processes prove the ability of strains of *A. fumigatus* not just to degrade but to utilize naphthalene and phenanthrene. The intermediates found match some of the chemicals previously reported in related investigations by other authors who studied members of various microbe classes. Despite the absence of genetic closeness, this suggests that these kinds of catabolic processes are more common and not just typical for fungi.

The growth and advancement of the two examined strains in a deficient mineral nutrient medium comprising unconventional organic substrates is essential for the formulation of a water purification technology that necessitates no further resources for their production.

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Authors' contributions

Z.A.: conceptualization; M.G.: methodology; Z.A.: validation; Y.M.: formal analysis; K.S., I.D., Y.M.: investigation; M.G.: data curation; Z.A.: writing - original draft preparation, review and editing.

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