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Bioremediation of Anthracene, Phenanthrene and Phenol by *Cunninghamella echinulata*

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Abstract

Oil-polluted areas contain large amounts of poly-aromatic hydrocarbons (PAHs) which represent severe hazards on the ambient environment. Fungal strains were isolated from different contaminated sites in the Mediterranean coast and middle Delta, Egypt and screened for PAHs degradation. Hydrocarbon-enriched media was used to isolate the anthracene-, phenanthrene- and phenol-degrading fungi. Thirteen fungal isolates showed high degradation activities for anthracene, phenanthrene and phenol. One isolate was selected possessing high degradation ability and incubation period and was identified based on 18S rDNA sequence. Partial sequence of 18S rDNA revealed that this isolate is identified as *Cunninghamella echinulata*. Laccase enzyme produced by this fungus was subsequently evaluated for its degradative ability towards PAHs mixtures. The percentage of degradation varied from 96.035% to 99.986% of the tested PAHs. After an incubation period of 45 days, 5% of PAHs gave the highest degradation activity among all concentrations used. The kinetics of PAHs degradation is presented along with the feasibility of using *Cunninghamella echinulata* for bioremediation

Keywords: 18S rDNA genes, biodegradation, environmental pollution, laccase, polycyclic aromatic hydrocarbons

Introduction

Environmental pollution is continuously increasing with the industrial development. Pollution causes many hazards for all organisms-including humans- such as carcinogenicity and toxicity. There has been increasing pollution with hydrocarbon compounds, many of which are considered to be potential health hazard [1]. Some hydrocarbon pollutants are polycyclic aromatic hydrocarbons (PAHs). Four classes of hydrocarbons are present: straight chain alkanes, branched chain alkanes, cycloalkanes and polyaromatic hydrocarbon [2]. PAHs are among the most harmful substances for human, marine and fresh water, soil fauna and flora. PAHs are a large group of organic compounds with two or more fused aromatic rings in linear, angular, or cluster arrangements. They have relatively low solubility in water, but are highly lipophilic [1].

Although PAHs can exist in over 100 different combinations, the most common are treated as a group of 15; they are: acenaphthene, acenaphtylene, anthracene, pyrene,

benzo(b)fluoranthene, benzo(ghi)perylene, benzo(k)fluoranthene, chrysene, dibenz(a,h)anthracene, Fluoranthene, Fluorine, indeno(1,2,3cd)pyrene, phenanthrene, pyrene [3].

Anthracene and phenanthrene are tri-cyclic aromatic hydrocarbons that are found at high concentrations in polycyclic aromatic hydrocarbon (PAH)-contaminated sediments, surface soils and waste sites. These hydrophobic contaminants are widely distributed in the environment, occurring as natural constituents of fossil fuels and their anthropogenic pyrolysis products [1]. Unlike the higher-molecular-weight PAHs, phenanthrene and anthracene do not pose a risk to human health, since they exhibit no genotoxic or carcinogenic effects. However, they have been shown to be toxic to fish and algae [4]. Phenol and phenolic compounds are well known components in a wide variety of waste waters including these from coal conversion processes, coking plants, petroleum refineries and several chemical industries, as pharmaceuticals, resin and dye manufactures [5].

PAHs have been reported to be degraded by some microorganisms in the soil [1]. The ability of fungi to transform a wide variety of hazardous chemicals is interesting as bioremediation strategy [6]. A large number of diverse microorganisms including bacteria, micro-algae, fungi, protozoa and actinomycetes are indigenous in most soils. Some microorganisms are capable of biodegrading petroleum hydrocarbons [7,8]. Petroleum hydrocarbon utilizing microorganisms have recently been isolated in large numbers form a wide variety of natural aquatic and terrestrial environments.

There are two essential characteristics that define hydrocarbon-oxidizing bacteria: 1) hydrocarbon-group-specific oxygenases and 2) mechanisms for optimizing contact between the bacterium and the hydrocarbon [9]. Some filamentous fungi possess some attributes that give them a good potential of degradation, since those microorganisms can ramify quickly on the substratum and through the secretion of extracellular enzymes. Moreover, the fungi are able to grow under an array of environmental conditions of stress, for example: environment with low pH values or poor in nutrients and with low water activity. Several authors have made lists containing bacterial and fungal genera that are able to degrade a wide spectrum of pollutants, proceeding from marine atmosphere as well as the soil. In accordance with several scientific publications, it can be pointed out that, amongst the filamentous fungi *Trichoderma* and *Mortierella* spp. are the most common ones isolated from the soil. *Aspergillus* and *Penicillium* spp. have frequently been isolated from marine and terrestrial environments.

The ability of fungi to transform a wide variety of hazardous chemicals has attracted interest in using them in bioremediation [10]. The white rot fungi are unique among eukaryotes for having evolved nonspecific methods for the degradation of lignin; curiously they do not use lignin as a carbon source for their growth [11]. Lignin degradation is, therefore, essentially a secondary metabolic process, not required for the main growth process. Lamar *et al*. [12] compared the abilities of three lignin-degrading fungi, *Phanerochaete chrysosporium*, *P. sordida* and *Tramates hirsuta* to degrade Pentachlorophenyl (PCP) and creosote in soil. Inoculation of soil with 10% (wt/wt) *Phanerochaete sordida* resulted in the greatest decrease of PCP and creosote. *P. sordida* was also most useful in the degradation of PAHs from soil. Davis *et al*. [13] showed that *P. sordida* was capable of degrading efficiently the three ring PAHs, but less efficiently the four-ring PAHs.

Laccase (Lac), manganese peroxidase (MnP) and lignin peroxidase (LiP) are the major lignin degradation enzymes of white-rot fungus [14]. Laccase is a multicopper oxidase which can degrade a variety of complex structures of xenobiotics.

The aim of this study was to isolate fungi with degradation activity of PHAs and to investigate the role of laccase in the degradation process.

Materials and methods

Study area

The study area is a part of Mediterranean coast and Delta of Egypt. It extends for about 500 km between Port Said to Alexandria and 500 Km to Delta (Port Said, 1; New Damietta, 2; Gamasa, 3; Balteem, 4; Kafr Zaiat, 5; and Alexandria, 6-8). From each location (1-5), composite samples were collected from two different sites, (a) and (b). Sample regions (a) and (b) are located on the tidal region and is about 2 km far from each other (horizontally), whereas (c) and (d) samples are sub-tidal and collected about 50 m2 away from a and b on land side. In Alexandria three localities were sampled viz; Abu Quir beach (6), Al-Montazah beach (7) and Al-Mandara beach (8).

Sample region (a) is on tidal site and c is far from it by 50 m2 landside.

Collection of soil samples

Composite soil samples were collected from 26 sites representing 8 selected localities along the Mediterranean Sea coast of Egypt and were separately transferred into clean plastic bags. Each sample was divided into two portions. The first portion was used directly for microbiological assay and then stored in cold conditions. The second portion was stored for physical and chemical analysis.

Soil characteristics

In 1:5 soil extracts (100 grams soil were used) the pH and the total soluble salts (TDS) were determined by using Corning pH-meter 215 and Sporule model 10 meter. The organic matter content (OC) was determined as recommended by Jackson [15]. Moisture content (MO) was evaluated as difference in weight between oven dry soil (105°C) and fresh soil. Water retention capacity (WRC) was assessed as in Piper [16]. Total pore space (porosity) is equal to the difference between the volume of soil in dry and wet conditions. Soil texture was determined by separation of different soil fractions by sieving method. The amount of each fraction was expressed as percentage of original weight used.

For determination of soil nutrients, dried soil material was ground into fine powder prior to digestion by sulphuric acid peroxide method of digestion [17] in a Kjeldal digestion flask. Total nitrogen (TN) was quantitatively determined by Markham micro-Kjeldal apparatus [18]. Ammonia–N (Nessler method) in the distillate was estimated colorimetrically at 450 nm by using Spectronic 20 D spectrophotometer [19]. Total petroleum hydrocarbons (TPH) were measured in ten grams of soil. The soil was dehydrated with Na_2SO_4 and mixed for 30 min with 10 ml of 1,1,2-trichlorotrifluoroethane; the TPH content of the filtrate was quantified as described in standard method [20]. Total phosphorus (%) was determined spectrophotometrically at 710 nm by modified method of John [21]. Na^+, K^+ and Ca^{+2} were determined in the previously prepared digest using a Flame Photometer type M7D.

Microbial enrichment and screening of isolated fungi

Adaptation and enrichment procedure involved an initial addition of 0.1g contaminated soil with PAHs from Mediterranean coast and Delta of Egypt with different concentrations of studied PAHs (100, 200, 300, 400, 500 mg 100 ml^{-1}) into the mineral Czapekdox broth medium as a sole carbon source (the PAHs was prepared with equal weights of anthracene, phenanthrene and phenol). Then the medium was maintained at 30±2ºC under shaking at 200 rpm. After 4 days, one ml of each sample was added and plated on Czapekdox agar medium. Then the plates were incubated for 7 days at 30±2ºC in the dark.

Fungal colonies were purified, and then screened on Czapekdox agar medium with the same previous concentrations of PAHs, and then growth was observed after 7 days and the diameter of growth was recorded.

A strain with high degradation activity was selected from the isolated fungi, and then was preserved on slants of the following medium: malt extract 3.0 g l^{-1}, yeast extract 3.0 g l^{-1}, peptone 5.0 g l^{-1}, glucose 10 g l^{-1} and agar 20 g l^{-1}, and stored at 4±1°C for further use.

Microorganism identification

The selected organism was identified using morphological methods by using various agar media for identification according to Arx Von [22], Barron [23], Domsch *et al*., [24], Ellis [25,26], Kozakiewicz [27], Raper and Fennel [28], Raper and Thom [29] and Rifai [30], in addition to different molecular techniques at Molecular Plant Pathology Department, Arid Lands Cultivation Research Institute (ALCRI), City for Scientific Researches and Technology Application, New Borg EL-Arab, Alexandria, Egypt.

Fingerprinting of the selected organism
DNA extraction

The genomic DNA was extracted using Wizard DNA Purification Kit (Promega, USA) following the protocol of genomic isolation fungi**.**

PCR amplification and sequencing of ITS- 18S rRNA region

The oligonucleotide primers used for amplification and sequencing of the ITS regions

including 18S rRNA were those described by White *et al.* [31]. The primers sequences ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5´-TCCTCCGCTTATTGATATGC-3´) were made by Bioneer (USA). Amplification reactions were performed in 25 µl containing 2.5 µl of each primer (20 picomole), 2.5 µl of genomic DNA (5 µg ml^{-1}) and one PCR bead (PuReTaq Ready-To-Go; Amersham Biosciences). PCR was performed using the initial denaturation at 94 ºC for 4 min, followed by 35 cycles at 94 ºC for 1 min, 55 ºC for 1 min and 72 ºC for 2 min, then final extension at 72 ºC for 10 min.

DNA sequencing and accession number

DNA sequence for the ITS gene was performed by Macrogene Company (Korea). The sequences were submitted to NCBI GenBank database and DNA sequences were aligned in sequin program (http://www.ncbi.nlm.nih.gov/sequin).

Alignment and phylogenetic analyses

BLAST [32] was performed for the obtained ITS DNA sequence to match the best similarities with other related ITSs on database. The best DNA sequence similarities with our ITS region were obtained from NCBI GenBank and aligned using CLUSTAL W [33]. Unalignable regions were excluded and the sequences from the same species and unidentified organisms were discarded. Finally, Phylogenetic tree analyses were conducted using *MEGA* version 4 [34].

Degradation test

The biodegradation was studied in the mineral medium containing (1%, 2%, 3%, 4% and 5% of PAHs mixture (100, 200, 300, 400, 500 mg 100 ml^{-1}) into the mineral Czapekdox broth medium as a sole carbon source, the mixture was prepared in equal weights of anthracene, phenanthrene and phenol and inoculated with the selected fungi**.**

HPLC analyses of the extracted PAHS for the determination of degradation

This method was according to Anderson and Henrysson [35]. At two-week interval samples, consisting of the whole flasks were collected and toluene (100 ml), was added to each flask, which were thoroughly mixed and placed in a water bath at 100°C for 3 hours. When the flasks had reached room temperature, 2 ml of the toluene mixture was removed, filtered with a 0.22 membrane using a glass syringe and used for the HPLC analyses.

Assay of laccase activity

One unit (U) of laccase activity was defined as the amount of enzyme oxidizing 1 µmol (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) ABTS min^{-1}. The assay mixture contained 0.2 mol l^{-1} acetic acid-sodium acetate buffer solution (pH 5.0), 5 mmol l^{-1} ABTS and a certain amount of enzyme solution. The oxidation of ABTS was followed by an absorbance increase of 0 – 3 min at 420 nm with spectrophotometer [36] .

Statistical analysis

Data generated were analyzed using (SPSS v.18). Statistically significant differences (at 0.05 level) among means of experiment results were evaluated by analysis of variance and means compared by one-way-ANOVA test Graphs were made using Sigma Plot v.18 software.

Results and Discussion

All the results of the soil samples are shown in Table 1. The examined soils are sandy, with very low nutrient content and slightly alkaline with pH ranged from 7.09-8.79 and with moderate to high electric conductivity; EC (2 - 80 mmhose cm^{-1}), organic carbon; OC (0.06 - 1.7 mg g^{-1} dry weight), total nitrogen; TN (0.004 - 0.20 mg g^{-1} dry weight), phosphorus; TP (0.001 - 0.012 %), sodium ion (23.09 - 57.8 %), potassium ion (1.2 - 3.61 %) and calcium ion (8 -112.17 %). The soil hydrocarbon content varied considerably between the different collected soils. All the samples were contaminated and the hydrocarbon content ranged from 0.025 to 0.422 mg l^{-1}. It was also obvious that sub-tidal samples are less in its constituents of EC, OC and TP than tidal ones. Conversely, TH and TN contents were higher in sub-tidal region than tidal one (Table 1).

Prince [37] reported that petroleum hydrocarbons are an excellent source of carbon and energy for certain microbes that could utilize and degrade them. However, hydrocarbons are deficient foods in that they do not contain significant concentrations of other nutrients, such as nitrogen and phosphorus, which are essential for microbial growth. Diesel oil is mainly

composed of simple unbranched n-alkanes and around 4% polyaromatic compounds [38]. Alkanes are normally toxic to microorganisms.

Thirteen fungal species were isolated from soil contaminated with hydrocarbon namely; *Alternaria alternata, Syncephalastrum racemosum, Aspergillus flavus, A. terreus, A. niger, A. tubingens, Trichoderma asperellum, Fusarium proliferatum, F. oxysporum, Pleurotus ostreatus, Rhizopus oryzae, Penicillium chrysogenum* and *Cunninghamella echinulata* (Table 2). These genera and their species have a worldwide distribution [30]. They are mostly isolated from oil-polluted soils [8,39] and reported to be active consumers of hydrocarbons and strongly lipolytic [40,41].

All species were very frequently (14-83 %) isolated except *Syncephalastrum racemosum* which was frequently (2%) isolated. The variation between them occurred only in the total colony count not in occurrence. This indicated that the soil properties may affect the abundance of species and not its existence. Spearman correlation indicated a significant negative correlation between hydrocarbon content (TH) and fungal count that is mean that the fungal abundance was affected by changes in TH level in soil. Alike, Reid *et al*. [42] stated that several factors including soil type and physico-chemical properties of the contaminant determine the fate and behaviour of PAHs in the soil.

Fungal isolation and screening on PAHs containing media was shown in Fig. 1-3. Fig. 1 illustrated that, the ability of isolated species to grow on phenol were variable. These were significantly increases as indicted by increasing the colony diameter. Consequently, *Cunninghamella* and *Penicillium chrysogenum,* were appear to be the best colonizers at 1% whereas their least growth was obtained at phenol concentration of 2%.

Table 1 Edaphic properties of soil samples collected from different sites along the Mediterranean coast and delta of Egypt.

Localities		Sample	Soil type	MC (%)	EC (mmhose cm^{-1})	OC (mg g^{-1} dry weight)	TN (mg g^{-1} dry weight)	TH (mg l^{-1})	pH	TP (%)	Na^{+} (%)	K^{+} (%)	Ca^{+2} (%)
Port said		1a	Sandy	3.33	30	0.75	0.019	0.237	7.09	0.005	42.2	3.0	16
		1c	Sandy	2.10	13	0.81	0.017	0.356	8.00	0.011	42.2	3.0	12
		1b	Sandy	2.05	28	0.75	0.004	0.255	8.00	0.006	31.1	2.4	10
		1d	Sandy	1.05	18	0.60	0.025	0.381	7.80	0.003	28.9	1.8	8
New Damietta		2a	Sandy	4.20	13	1.10	0.200	0.294	8.79	0.011	24.2	2.9	22
		2c	Sandy	4.30	14	1.20	0.030	0.422	8.08	0.001	24.8	1.4	33
		2b	Sandy	2.60	17	1.30	0.050	0.034	8.22	0.002	23.1	1.8	12
		2d	Sandy	2.80	10	1.70	0.010	0.366	7.90	0.001	30.2	1.9	17
Gamasa		3a	Sandy	3.37	30	0.99	0.019	0.334	8.19	0.002	42.2	3.0	22
		3c	Sandy	0.01	13	0.06	0.017	0.237	8.14	0.005	42.2	3.0	16
		3b	Sandy	0.24	28	0.06	0.004	0.356	8.21	0.011	31.1	2.4	12
		3d	Sandy	0.10	18	1.20	0.025	0.255	7.90	0.006	28.9	1.8	10
Kafr Zaiat		4a	Sandy	0.65	26	0.24	0.039	0.381	7.90	0.003	57.8	2.4	8
		4c	Sandy	0.52	80	1.05	0.037	0.391	7.90	0.005	37.8	1.2	8
		4b	Sandy	2.08	30	1.47	0.019	0.039	7.80	0.007	26.7	1.2	8
		4d	Sandy	1.30	21	1.11	0.009	0.025	8.00	0.012	28.9	1.2	36
Balteem		5a	Sandy	4.20	42	1.14	0.098	0.393	8.38	0.002	28.9	3.6	30
		5c	Sandy	2.60	2.0	0.93	0.036	0.310	8.03	0.004	24.5	1.8	12
		5b	Sandy	3.44	38	1.65	0.009	0.262	7.90	0.005	33.4	2.4	10
		5d	Sandy	2.56	47	0.36	0.049	0.293	7.80	0.007	28.9	1.8	18
Alexandria	Abu Quir	6a	Sandy	7.21	35	0.54	0.023	0.310	8.09	0.003	42.2	3.6	32
		6c	Sandy	5.33	29	0.96	0.015	0.273	8.03	0.002	33.6	2.4	64
	Al-Montazah	7a	Sandy	4.77	23	0.75	0.007	0.293	7.88	0.003	31.1	1.8	84
		7c	Sandy	2.90	4.0	0.81	0.023	0.310	8.03	0.002	35.6	1.8	84
	Al-Mandara	8a	Sandy	7.30	37	0.75	0.022	0.388	8.03	0.002	51.1	2.4	112
		8c	Sandy	4.30	16	0.81	0.017	0.252	7.85	0.002	53.4	2.4	22

a and b tidal samples; c and d, sub-tidal.

Table 2 Record of fungi isolated from sandy beaches along the Mediterranean coast and Delta of Egypt.

	Tidal samples		Sub-tidal samples	
	Total count	% Occurrence	Total count	% Occurrence
Alternaria alternata	68	68	54	54
Aspergillus flavus	156	83	181	79
A. niger	32	43	51	52
A. terreus	49	42	74	29
A. tubingens	18	21	9	18
Cunninghamella echinulata	43	68	56	64
Fusarium oxysporum	36	50	56	36
F. proliferatum	16	36	18	39
Penicillium chrysogenum	41	61	71	68
Pleurotus ostreatus	7	14	11	18
Rhizopus oryzae	41	61	74	29
Syncephalastrum racemosum	2	4	1	2
Trichoderma asperellum	40	57	35	46

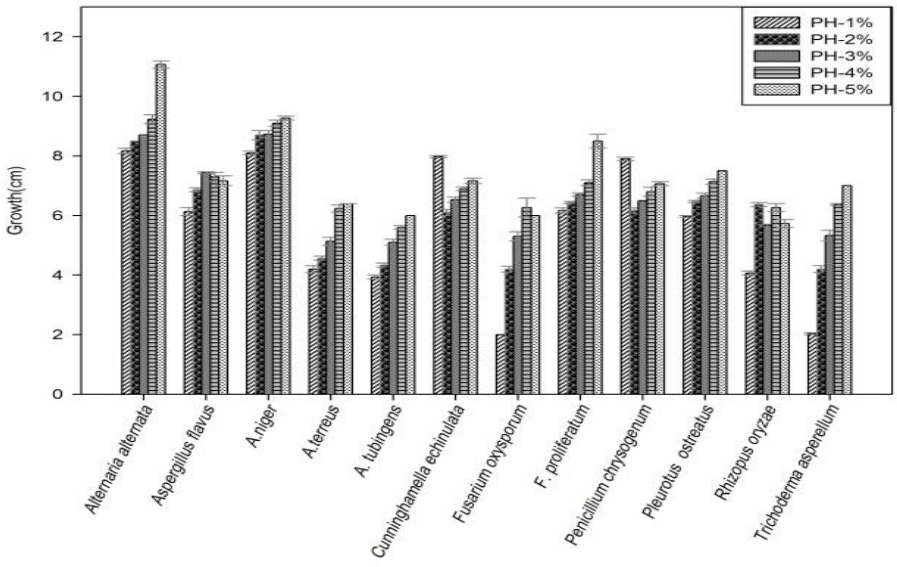

Fig. 1 The growth of the isolated fungi on the phenol (PH). Fungi were grown on Czapekdox medium containing PH as a sole carbon source for 7 days at 30°C. Bars are means of colonies diameters (cm) ±SE.

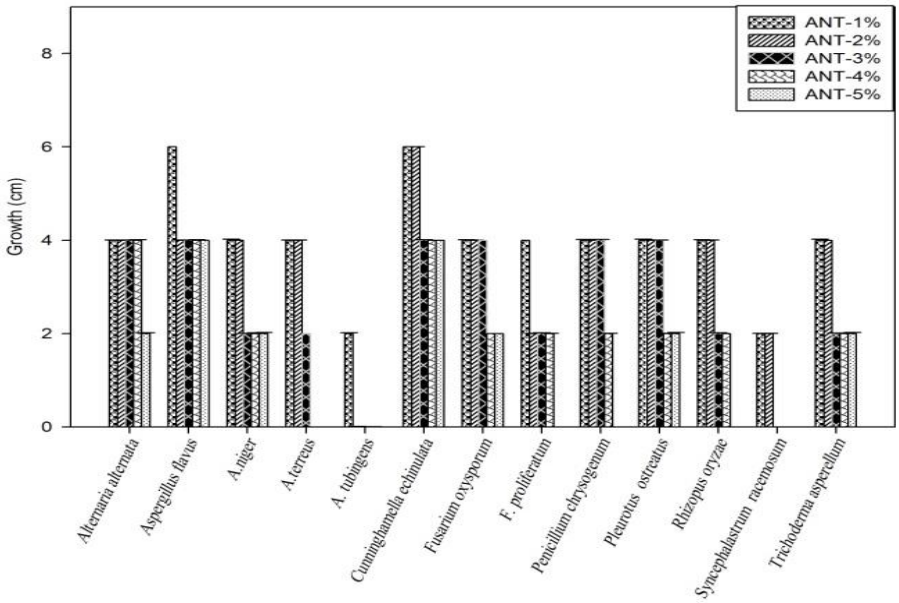

Fig. 2 The growth of the isolated fungi on Anthracene (Ant). Fungi were grown on Czapekdox medium containing Ant as a sole carbon source for 7 days at 30°C. Bars are means of colonies diameters (cm) ±SE.

On the contrary, *Rhizopus oryzae* showed their highest growth at phenol concentration of 2% and its least growth was obtained at 1% concentration. Moreover, *Pleurotus ostreatus, Fusarium oxysporum, F. proliferatum, Trichoderma asperellum, Aspergillus tubingens, A. niger, A. terreus, A. flavus* and *Alternaria alternata*, showed the highest growth at phenol concentration of 5%, whereas their reduced growth was obtained at phenol concentration of 1%.

The results in Fig. 2 point out that, the ability of tested fungi to grow on anthracene were variable as recognized by increases in colony diameter. Therefore, *Cunninghamella*, *Rhizopus oryzae, Penicillium chrysogenum, Pleurotus ostreatus, Fusarium oxysporum, F. proliferatum, Trichoderma asperellum, A. tubingens, A. niger, A. terreus, Aspergillus flavus* and *Alternaria alternate* were the best colonizers at 1%.

Fig. 3 illustrates that, the ability of fungi to grow on phenanthrene were also variable and significantly increases as recognized by increases in colony diameter. *Cunninghamella*, *Penicillium chrysogenum, Pleurotus ostreatus, Fusarium oxysporum, F. proliferatum, Trichoderma asperellum, A. tubingens, A. niger, A. terreus, Aspergillus flavus* and *Alternaria alternate*, were appear to be the best colonizers at 1% concentration of phenanthrene. While *Rhizopus oryzae* gave the same growth value with different concentrations of phenanthrene.

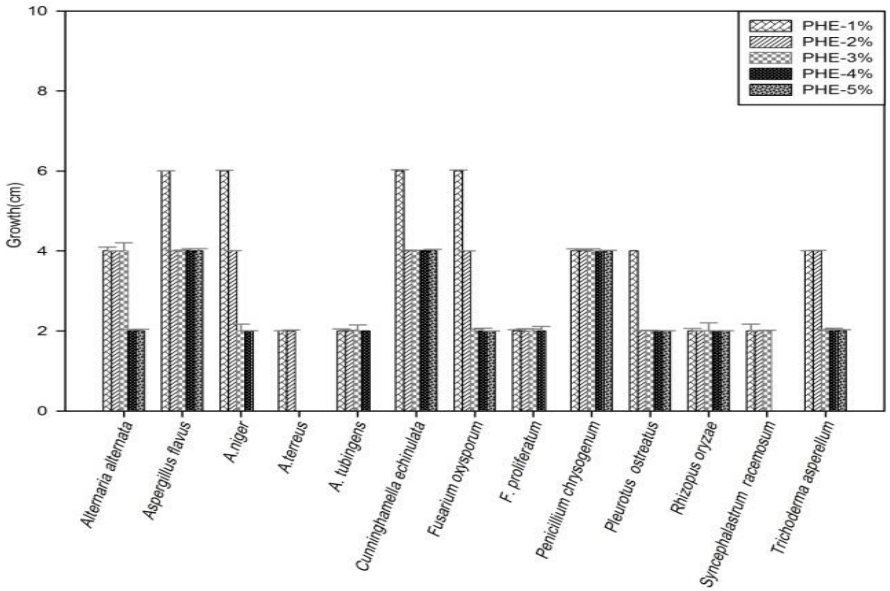

Fig. 3 The growth of the isolated fungi on phenanthrene (PHE). Fungi were grown on Czapekdox medium containing PHE as a sole carbon source for 7 days at 30°C. Bars are means of colonies diameters (cm) ±SE.

At the end of this part *Cunninghamella echinulata* was selected for further studies according to their high degradation activity towards PAHs. The fungus was identified as based on morphological characteristics. The DNA sequence of its rDNA is shown in Fig. 4. The obtained 475 base nucleotide sequence was compared with the corresponding 18s ribosomal RNA gene in NCBI to obtain the Phylogenetic tree shown in the Fig. 5.

Cunninghamella echinulata was found to be able to degrade three-ring polyaromatic hydrocarbons (anthracene and phenanthrene) and phenol aromatic compounds. The percentage of degradation varied from 96.035% to 99.986% of the tested PAHs (Table 3). The fungus produces laccase enzyme that is able to degrade PAHs compounds. For biodegradation of phenanthrene tested at levels of 1, 2, 3, 4 and 5% the fungus was able to degrade them to levels of 99.9, 99.4, 99.98, 99.7 and 99.7 % respectively. Alike, *Cu. echinulata* was able to degrade 99.98375, 99.4712, 99.97874, 99.7685 and 99.8435 % for the anthracene concentrations of 1, 2, 3, 4 and 5%, respectively. Phenol was tested at the same levels (1, 2, 3, 4 and 5%) that also degraded to 96.035, 99.4858, 99.7578, 99.8184 and 99.1286 % respectively. The percentage of degradation varied from 96.035% to 99.986% of the tested PAHs (Table 2). Moreover, the experiments conducted with PAHs (mixture) as a sole carbon source indicated that the organism was capable of utilizing this material as a source of carbon for its growth.

Enzyme activities are measures of microbial activity in soil [43] and are indicative of the onset of hydrocarbon biodegradation [44]. The data revealed that *Cunninghamella echinulata* has the

properties of an efficient phenol, phenanthrene and anthracene degrading microorganism. The efficiency of a certain catabolic pathway often depends on the properties of the involved key enzyme(s). Laccase activity was measured frequently as indicators of the microbial activity during the bioremediation processes in this investigation. The biological activity increased during the phase of the highest degradation activity.

Table 3 The percentage of the degradation of *Cunninghamella* grown in1%-5% of mixing polyhydrocarbon for 45 days.

Initial conc.	phenanthrene		anthracene		phenol	
gm l^{-1}	% degradation	residue	% degradation	residue	% degradation	residue
1	99.9869	0.00013	99.9837	0.000162	96.035	0.03965
2	99.4213	0.00578	99.4712	0.005288	99.485	0.00514
3	99.9853	0.00014	99.9787	0.000212	99.757	0.00242
4	99.7072	0.00292	99.7685	0.002315	99.818	0.00181
5	99.8603	0.00139	99.8435	0.001565	99.128	0.00871

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ACATCTCCCTCAATCCTATTTTTTTATAGAGAATGAGATCAGAGATAAATTATAAATGGTCCTGGGTAAGCTGTGCTATA
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Fig. 4 18s ribosomal RNA gene among the selected strains (unknown).

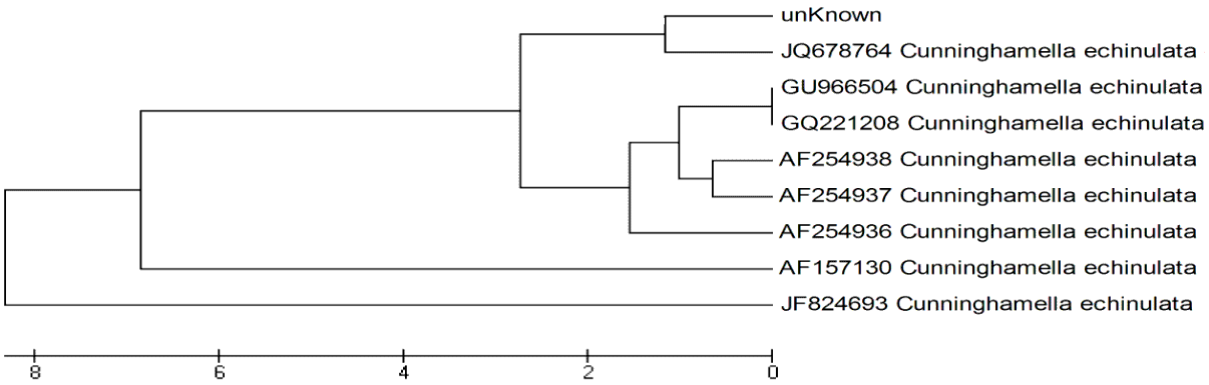

Fig. 5 Phylogenetic relationships among the selected strains (unknown) based on sequence analysis and the most closely related fungal species.

The specific laccase activities in cell free extract and culture media was determined (Fig. 6). Fig. 6 and Table 4 illustrates that the laccase activity was increased gradually until the end of the experiment (45 days of incubation period) with the highest activity values of 0.3133, 0.6767, 0.7800, 1.5233 and 1.5833 at 1, 2, 3, 4 and 5%of the mixture of PAHs. There was a significant difference between laccase activity (LSD at 0.05 Level). Therefore, the increased of laccase activity can be explained for the substance conversions and mineralization of the substrates. Similar results were observed by Waarde *et al.* [45] and Margesin and Schinner [46,47]. Also the previous authors noted that there is an increase of laccase activity and they explained this decrease of biological activities as the lack of growth factors, accumulation of inhibiting metabolites and of recalcitrant long-

chain alkanes, high-branched aromatics and condensates [46].

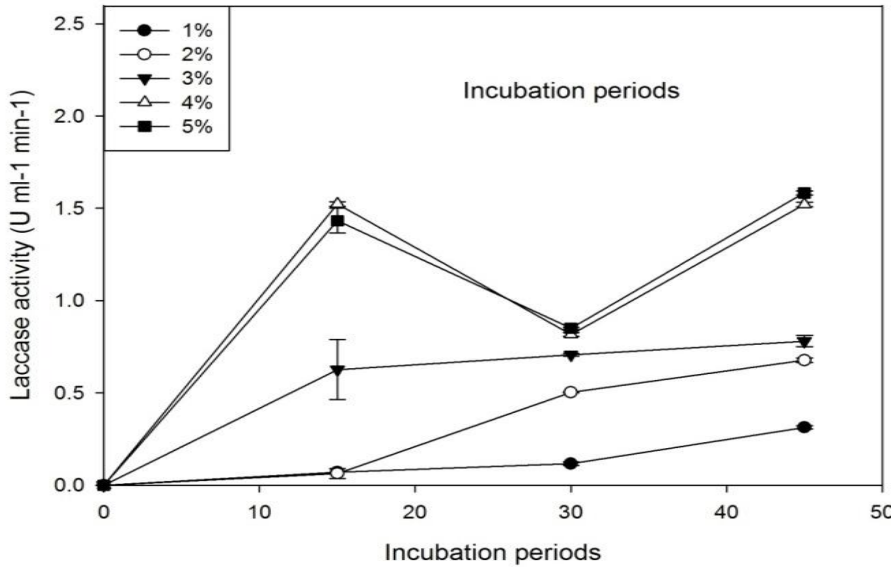

Fig. 6 laccase activity of *Cunninghamella* grown in1%-5% of mixing polyhydrocarbon (PH, ANT AND PHE) for 45 days, Data are represented as means of three replicates with standard errors.

Table 4 laccase activity of *Cunninghamella* grown in1%-5% of mixing polyhydrocarbon (PH, ANT AND PHE) for 45 days, Data are represented as means of three replicates with standard errors. Statistics was done using ANOVA (one way) at $p \leq 0.05$,

Time	Conc.				
	% 1	% 2	% 3	% 4	% 5
15	0.0700± 0.00[a]	0.0633± 0.026[a]	0.6267±0.16 [a]	1.5233± 0.01[a]	1.4333±0.06[a]
30	0.1167± 8.82e-3[b]	0.5033± 3.33e-3[b]	0.7067±6.67e-3[b]	0.8167± 8.82e-3[b]	0.8500±5.78e-3[a]
45	0.3133± 8.82e-3[c]	0.6767±0.012 [c]	0.7800±0.03 [c]	1.5200±0.01 [c]	1.5833± 0.01[a]

Different letters in the same column refers to significant difference

The efficiency of a certain catabolic pathway often depends on the properties of the involved key enzyme(s). Little is known about phenol metabolism in mycelial fungi. The specific laccase activities in cell free extract and culture media are shown in Fig. 6. This mechanism may contribute to metabolic adaptation of ubiquitous fungus found in nature, such as *Graphium*, *Aspergillus* and *Penicillium*, exposed to xenobiotic and aromatic compounds.

Finally, we can conclude that *Cunninghamella echinulata* showed a high ability for PAHs degradation and high activity of laccase. So, it is recommended to be use as a bioremediant of polluted areas.

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الملخص العربى

المعالجة البيولوجية لمركبات الانثراسين والفينانثرين والفينول بفطرة كانينجهاميلا اكانيولاتا

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تحتوى المناطق الملوثة بزيت البترول على كميات كبيرة من الهيدروكربونات عديدة الحلقات والتى تمثل خطرا كبيرا على البيئة. تم عزل العديد من الفطريات من مناطق ملوثه بزيت البترول على طول البحر الأبيض المتوسط وفى وسط الدلتا فى مصر حيث تم اختبار قدرتهم على تحليل المركبات الهيدروكربونية عديدة الحلقات باستخدام بيئات تحتوى على مركبات الانثراسين والفينانثرين والفينول لعزل تلك الفطريات التى لها القدرة على التحليل وكذلك قمنا بعمل تأقلم لهذه العزلات الفطرية عند تركيزات مختلفة من الأنثراثين والفنانثرين والفينول (1-5%). تم اختيار عزلة واحدة ذات قدرة عالية على تحليل الهيدروكربونات وتم تعريفها عن طريق 18S rDNA عرفت أنها كانينجهاميلا اكانيولاتا كما تم اختيار إنتاج إنزيم اللاكيز من الفطرة المعزولة وقدرتها على تحليل الهيدروكربونات بواسطته. وكانت نسبته على تحليل تلك المركبات حوالى 96.035% الى 99.986% وكانت أعلى نسبه للتحليل بعد فترة 45 يوم وتركيز 5% من الهيدروكربونات. وهذا يدل على قدره فطرة كانينجهاميلا اكانيولاتا على تحليل تلك المركبات بسهولة.