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# Biodegradation of petroleum hydrocarbons by filamentous fungi (*Aspergillus ustus* and *Purpureocillium lilacinum*) isolated from used engine oil contaminated soil

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ABSTRACT

The use of microorganisms for remediation and restoration of hydrocarbons contaminated soils is an effective and economic solution. The current study aims to find out efficient telluric filamentous fungi to degrade petroleum hydrocarbons pollutants. Six fungal strains were isolated from used engine (UE) oil contaminated soil. Fungi were screened for their ability to degrade crude oil, diesel and UE oil using 2.6-dichlorophenol indophenol (DCPIP). Two isolates were selected, identified and registered at NCBI as *Aspergillus ustus* HM3.aaa and *Purpureocillium lilacinum* HM4.aaa. Fungi were tested for their tolerance to different concentration of petroleum oils using radial growth diameter assay. Hydrocarbons removal percentage was evaluated gravimetrically. The degradation kinetic of crude oil was studied at a time interval of 10 days. *A.ustus* was the most tolerant fungi to high concentration of petroleum oils in solid medium. Quantitative analysis showed that crude oil was the most degraded oil by both isolate; *P. lilacinium* and *A. ustus* removed 44.55% and 30.43% of crude oil, respectively. The two fungi were able to degrade, respectively, 27.66 and 21.27% of diesel and 14.39 and 16.00% of UE oil. As compared to the controls, these fungi accumulated high biomass in liquid medium with all petroleum oils. Likewise, crude oil removal rate constant (K) and half-lives ($t_{1/2}$) were 0.02 $day^{-1}$, 34.66 day and 0.015 $day^{-1}$, 46.21 day for *P. lilacinium* and *A. ustus*, respectively. The selected fungi appear interesting for petroleum oils biodegradation and their application for soil bioremediation require scale-up studies.



## 1. Introduction

The continuous increase in world oil demand has increased the oil production, refining and distribution activities which lead to the pouring of a huge amount of petroleum hydrocarbons into the environment. The impact of oil spills accidents on soil ecosystem is hazardous; the presence of hydrocarbons in the soil inhibited seed germination and reduced plant growth [1]. The persistence nature of crude oil affects the soil quality by changing physical, physiological, biochemical properties and intrinsic heterogeneous microbial diversity [2]. Diesel fuel physically impedes water and oxygen transfer between the seed and the surrounding soil environment which leads to delaying seed emergence and reducing percentage germination of plants [3]. Used engine oil was found to exert a great toxicity toward earthworm survival and soil enzymatic activities [4]. Generally, Human health and ecosystem safety are directly influenced by accumulation of petroleum products in the environment [5]. Thus, the high impact of oil spillage on soil environment requires effective remediation methods of these contaminants.

A variety of remediation technologies have been developed, most of them are chemical, thermal and physical-chemical remediation technologies as they may be a quick and simple solution to remove oil contaminants. However, many disadvantages are associated to these methods which require mechanical and labour intensive methods. In addition, these mechanical methods are tedious, time consuming and are only capable of removing the oil contaminants up to a certain extent, leaving behind a large amount of oil adsorbed in the soil [6]. Bioremediation is a way of cleaning up contaminated environments by exploiting the diverse metabolic abilities of microorganisms to convert contaminants to harmless products by mineralization, generation of carbon (IV) oxide and water, or by conversion into microbial biomass [7].

Fungi have received more attention in the past two decades for their bioremediation potential. They are known to degrade a variety of materials and compounds, process known as *mycodegradation* [8–11]. Fungi constitute an important part of the soil biomass and play vital roles in nutrient cycling and biotic interactions through their mycelial networks [12]. Fungi are equipped with a well-developed enzymatic system that gives them the ability to grow on a broad range of natural as well as synthetic substrates. They secrete different extracellular enzymes into their peripheral environment and degrade various substrates to small molecules that can be absorbed by and metabolized in their cells [7].

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The mycelial structure of these microorganisms is another advantage for bioremediation with its invasive nature which allows the investigation of a large volume of contaminated sites with a very large exchange surface [13]. Numerous studies were focused on the capacity of telluric fungal strains to degrade petroleum hydrocarbons [14–16]. These strains are in fact more adapted to the environmental conditions of the soil, which theoretically makes them more capable of undergoing competition from the other organisms present. Particularly saprotrophic strains that decompose organic matter containing complex molecules such as lignin and thus have potential for use in bioremediation process [17]. The isolation of cultivable fungi from oil polluted soils provides fungal strains more adapted to polluted conditions than strains directly obtained from culture collections [18].

At present, there is considerable interest on identification of microorganisms that degrade petroleum hydrocarbons, particularly, the recalcitrant fractions of crude oil and lubricants, because they are the critical components in influencing the success rate of bioremediation [19]. Although, many research focused on mycoremediation of individual components in petroleum hydrocarbons particularly polycyclic aromatic hydrocarbons (PAHs) due to their recalcitrant, toxic, mutagenic and often carcinogenic properties [20–22], more information about total petroleum hydrocarbons (TPHs) degradation potential of indigenous fungal strains remain necessary before establishing a successful field-level bioremediation experiments.

Therefore, an attempt has been made to isolate more potent fungal strains effective in petroleum hydrocarbons biodegradation. The aim of this study was to investigate the ability of telluric filamentous fungal isolates to grow on crude oil, diesel and used engine oil as the sole carbon source and to evaluate their degradation potential in liquid culture. The selected fungi are likely to be useful in bioremediation of oil contaminated sites.

## 2. Material and methods

### 2.1. Sample collection and soil contamination

Uncontaminated soil samples were collected from ground surface (0–15 cm in depth) in Kherrouba, Mostaganem State, Algeria. The collected soil with no previous history of hydrocarbons contamination was air-dried and sieved through a 2 mm mesh for homogenizing and removal of large particles. Then, selected physical and chemical characteristics of this soil were determined (Table 1). To artificially contaminate the soil for the experiment, 4 kg of soil were placed in plastic vessels with a volume of about 3000 $cm^3$, and 100 ml of UE oil was added and thoroughly mixed to make uniform contaminated soil. A control soil without hydrocarbons contamination was prepared in the same conditions [23]. The plastic vessels were incubated outdoor exposed to environment conditions for three months.

### 2.2. Strain isolation

After 3 months of incubation, the enumeration of heterotrophic fungi in the oil polluted soil and control was carried out using dilution plate count method. 1 g of soil was added to 9 ml sterilized distilled water, mixed thoroughly by vortex, then serial dilutions ($10^{-5}$) were done and 0.5 ml of each dilution was inoculated on potato dextrose agar (PDA) dishes added with chloramphenicol as antibiotic (250 mg/l). Petri dishes were incubated at 25 °C for 5 days. Fungal colonies were selected and transferred to new PDA plates until pure colonies were obtained. The pure isolates were identified according to their cultural and morphological characteristics using identification keys [24–26].

### 2.3. Preliminary evaluation of hydrocarbons degradation ability

Fungi were initially screened for utilization of crude oil, diesel and UE oil using modified technique from Hanson et al. [27], based on the redox indicator 2.6-dichlorophenol indophenol (DCPIP). Therefore, Bushnell-Haas [28] (BH) broth medium: $MgSO_4$ (0.2 g/l), $CaCl_2$ (0.02 g/l), $KH_2PO_4$ (1 g/l), $K_2HPO_4$ (1 g/l), $NH_4NO_3$ (1 g/l) and $FeCl_2$ (0.05 g/l), distilled water (1000 ml), initial pH adjusted to 7.0, was used for the screening test. Two culture plugs (8 mm diameter) were picked from the edge of actively growing colony of each fungus on PDA plate and transferred into 50 ml BH medium using 250 ml conical flask. 1% (v/v) crude oil, diesel or UE oil and 0.016 mg/ml of redox indicator were added to BH medium. A control set without any fungi was also prepared. All flasks were incubated for 2 weeks at 30 °C under constant rotary shaking of 110 rpm. The color change of the DCPIP indicator from blue (oxidized form) to colorless (reduced form) indicates the ability of fungi to degrade hydrocarbons [29]. According to their positive responses to the redox indicator DCPIP, fungal isolates were subjected to further tests in this study.

**Table 1**
Physicochemical properties of the soil used in this study.

| Soil property | values |
|---|---|
| pH | 7.35 |
| Organic carbon (%) | 0.48 |
| Total N (%) | 0.0448 |
| P (mg/kg) | 3.126 |
| Ca (mg/kg) | 4548.8 |
| Mg (mg/kg) | 134.16 |
| Na (mg/kg) | 371.68 |
| K (mg/kg) | 95.55 |
| Silt (%) | 5.0 |
| Clay (%) | 10.8 |
| Sand (%) | 84.2 |

### 2.4. Molecular identification of oil-degraded isolates

Genomic DNA was extracted from fungal colonies growing in PDA plates for 7 days, using the DNeasy Plant Mini Kit (Qiagen), in accordance to manufacturer's instructions (QIAGEN, Hilden, Germany). DNA extracts were assessed using a Nano drop 1000 Spectrophotometer (Thermo Scientific, USA) and stored at −20 °C for further use. The extracted fungal DNA was used for PCR amplification of the internal transcribed spacer (ITS) region of nuclear rDNA using specific fungal primers ITS1 (5′- TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCC GCTTATTGATATGC-3′) [30,31]. The PCR reactions were performed in 25 μl volumes containing 2 μl of genomic DNA, 1 X Promega Taq Buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 mM of each primer and 1 U of Taq DNA polymerase (Promega). Amplification was carried out in Thermal Cycler (Icycler Biorad, USA) at 95 °C for 5 min followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s and final extension at 72 °C for 7 min. PCR products were electrophoresed in 1.5% (w/v) agarose gels then sequenced after the purification, and sequence similarities were obtained using the blast tool from National Center for Biotechnology Information (NCBI).

### 2.5. Petroleum hydrocarbons tolerance test in solid medium

Tolerance test of selected isolates toward different concentrations of crude oil, diesel, and UE oil was carried out on BH agar surface culture (pH 7.0) in Petri dishes of 9 cm in diameter. The concentrations used in this experiment were 2, 4 and 6% (v/v). The dishes were prepared by adding each hydrocarbon to warm BH medium and the solution was thoroughly mixed right before adding to the plates. A suspension of spores was prepared in warm BH medium by fungal spores harvested from the edge of an actively growing culture on PDA plate. The inoculated solution was vortexed in high speed and used to inoculate the center of the BH agar plate. Each treatment was prepared in duplicate and the plates were incubated at 30 °C. Plates without hydrocarbons and

inoculated with each of the strains were used as controls. Fungi mycelia extension on the plates was measured on a daily basis and also macroscopic observations about the morphology of the colonies were made and compared against the control plates to assess their capacity to tolerate different concentrations of each petroleum oil in surface culture [14,20]. Mycelium growth rate was estimated as the slope of a linear regression of colony radius versus time during the phase of linear growth [32]. Linear regression fitted data well with $R^2$ ranging from 0.98 to 0.59 (median $R^2 = 0.93$) [33].

### 2.6. Crude oil, diesel and UE oil biodegradation assay

Degradation experiments were performed in 250 ml flasks containing 100 ml of BH medium (pH 7.0) and 2% (*v*/v) of crude oil or diesel and 50 ml of BH with 1% (v/v) of UE oil as the only carbon source, separately. Prior to adding petroleum oils, media were sterilized by autoclave at 121 °C for 20 min. Mycelia plugs of selected fungi (three 8 mm discs) were cut from the edge of an actively growing colony on PDA and inoculated into each flask. Then flasks were incubated at 30 °C for 40 days. Abiotic control experiments were prepared by incubating each hydrocarbon in BH without inoculum. All assays were performed in triplicate [16]. The flasks were shaken manually at regular interval throughout the time of the experiment to facilitate oil-cell phase contact [8,34].

### 2.7. TPHs extraction and gravimetric analysis

The residual petroleum oils in all flasks were recovered through liquid-liquid extraction. Crude oil and diesel were sequentially extracted with 40 ml aliquots of petroleum ether:acetone (1:1) in a separating funnel and shaken vigorously to separate hydrocarbons adsorbed on mycelia. After that, the contents were allowed to settle; the top layer containing petroleum ether mixed with crude oil or diesel and acetone was taken out and the solvent was then evaporated under reduced pressure [35]. Residual UE oil was removed from the culture with toluene using the same previous steps. The residual oil was accurately weighed and quantified gravimetrically. The percentage of degradation was then calculated as follows [36]:

$$\%\boldsymbol{degradation} = \frac{\boldsymbol{a}-\boldsymbol{b}}{\boldsymbol{a}} \times \mathbf{100} \quad (1)$$

where: a is the mass of the oil in the control, b is the mass of the oil remaining after treatment

### 2.8. Fungal biomass gain via biodegradation

After measuring the amount of oil degradation, the fungal biomass in each flask was harvested using a filter paper (Whatman No.1) and then dried at 60 °C until constant weight was reached. Three flasks with corresponding inoculum but without petroleum oils were retained as controls. Gain in biomass under each treatment and the corresponding control was recorded; and considered to be due to biodegradation activity of the fungus [37].

### 2.9. Crude oil degradation kinetics

To provide an estimation of hydrocarbons biodegradation over time, flasks with 100 ml BH medium (pH 7.0) and 2% (*v*/v) crude oil were inoculated with selected strains and incubated at 30 °C. Inoculated flasks were sacrificed after 0, 10, 20, 30 and 40 days and the amount of remained oil and mycelium biomass accumulation were measured.

Biodegradation data were fitted to the first-order kinetics model [38,39]:

$$\boldsymbol{C_t} = \boldsymbol{C_0 e^{-kt}} \quad (2)$$

where $C_t$ is the residual hydrocarbons concentration (g/l) at time t, $C_0$ is the initial hydrocarbons concentration, k is the first-order kinetic biodegradation constant ($day^{-1}$) and t is time (day).

Biodegradation half-life time ($t_{1/2}$) of crude oil was calculated as follow:

$$\boldsymbol{t_{1/2}} = \frac{\ln 2}{\boldsymbol{k}} \quad (3)$$

### 2.10. Statistical analysis

Data obtained in this study were analyzed using IBM SPSS Statistics 25 software. Mean of the replicates and standard deviation was calculated. One-way analysis of variance (ANOVA) at $p \leq 0.05$ level of significance and Duncan's multiple range tests were applied for comparison between the means.

## 3. Results and discussion

### 3.1. Fungal isolation and identification

The aim of mixing used engine oil with soil was to increase the number of hydrocarbon-utilizing fungi and to promote the appearance of fungal species with ability to degrade petroleum oil products, as UE oil contains large amounts of hydrocarbons including toxic PAHs [40]. Three month after the experiment, the average counts of total heterotrophic fungi in the soil treated with oil were expressed as $4.1 \times 10^6$ CFU/g soil while $3.1 \times 10^3$ CFU/g soil were counted in the control soil. Results indicate that the population density of heterotrophs in the polluted soil was higher than in non-polluted soil (control). Concentration of total heterotrophic microorganisms has been found to rise rapidly and significantly after petroleum oil inputs in soil and marine sediments [41]. Atlas [42], reported that the presence of oil pollution increase soil populations of hydrocarbon-degraders from 1% to typically 10% of the community.

Six different isolates were obtained from oil-polluted soil. Macroscopic and microscopic examination of the isolates showed typical structures like members of the following genera: *Aspergillus* (2 species), *Penicillium*, *Fusarium*, *Peacilomyces* and *Alternaria*. These genera include the most frequently isolated species from hydrocarbons contaminated soils [22,43,44].

### 3.2. Screening of hydrocarbons degradation ability through DCPIP assay

The screening method adopted in this study allowed us to quickly select the fungal isolates that have ability for crude oil, diesel or UE oil oxidation. Three main indicators guide to estimate the biodegradation ability of fungi: change in color of the media from blue to colorless, reduction in petroleum oil quantity and the appearance of mycelial growth in the medium [45]. Four isolates showed efficient biodegradation of hydrocarbons, since change in color of DCPIP was observed with almost all tested oils, crude oil is mostly being used by these fungi (Table 2). Complete change of color and strong fungal proliferation was observed in media containing crude oil and UE oil with isolates denoted HM3 and HM4. These isolates were retained for further analysis.

### 3.3. Molecular identification of the selected isolates

The partial sequence of the 18S rRNA gene from the two selected isolates were analyzed and compared with sequence data in the GenBank using NCBI BLAST. Results of sequence analysis supplemented with morphological observations allowed identifying the isolate HM3 as *Aspergillus ustus* HM3.aaa and HM4 as *Purpureocillium lilacinum* HM4.aaa

**Table 2**
Decolourization magnitude of 2.6-DCPIP and visual estimation of fungal growth after 14 days incubation with different petroleum oils (crude oil, diesel and UE oil).

| Isolate | Crude oil | | Diesel | | UE oil | |
|---|---|---|---|---|---|---|
| | decolourization | Mycelial growth | decolourization | Mycelial growth | decolourization | Mycelial growth |
| HM1 | ++ | ++ | +++ | + | − | − |
| HM2 | +++ | + | ++ | ++ | +++ | +++ |
| HM3 | +++ | +++ | ++ | ++ | ++ | +++ |
| HM4 | +++ | +++ | ++ | ++ | +++ | +++ |
| HM5 | + | + | + | − | − | − |
| HM6 | − | − | − | − | − | − |
| Control | − | − | − | − | − | − |

Indicator change color. -: no change in color, +: weak, ++: medium, +++: complete decolourization.
Mycelial growth. -: no visual growth, +: weak, ++: medium, +++: strong.

and affiliated with accession numbers of MT192486 and MT192485, respectively (Fig. 1). Both isolates belongs to phylum Ascomycota. *P. lilacinum*, previously named as *Paecilomyces lilacinus*, is a common soil saprotrophic filamentous fungus [46]. In an earlier study, the growth of the genus *Peacilomyces* was induced after oil waste application to the soil while it was almost absent in the control area [47]. *P. lilacinum* was isolated from PAHs contaminated soil in Austria [48]. Species of Aspergillus are widely isolated from hydrocarbons polluted environments [15,49,50]. Husaini et al. [14] had successfully isolated three *Aspergillus* species from soil contaminated with used motor oil.

### 3.4. Hydrocarbons tolerance test

Many research studies on hydrocarbons and their derivatives degrading fungi have adopted the colony growth rate evaluation method for screening the growth and tolerance of filamentous fungal isolates [14,15,51]. The ability of tested fungi to withstand high concentrations of contaminants is an essential feature that must be taken into consideration when selecting strains for bioaugmentation [52]. The selected isolates were screened for their capacity to grow on 0, 2, 4 and 6% of all studied petroleum oil in surface culture. Radial growth rate of each strain, in the presence of different concentrations of hydrocarbons, are shown and examined by variance analysis for any significant differences with respect to the control (Table 3). The two isolates showed distinct behavior toward tolerance of the tested petroleum oils which can be attributed to the intrinsic physiological differences between genus and species, and their specific growth requirements. *A. ustus* tolerated every concentrations of all petroleum oil employed in this experiment and its growth rates were faster than that of the control plate (0%). Maximum tolerance of *A. ustus* was at 2% crude oil where the growth rate ($0.94$ cm day$^{-1}$) showed significant difference ($p = 0.041$) compared to the control while high concentration of hydrocarbons (6%) had no significant inhibitory effect on growth rate of this isolate although it exhibited slow growth with crude and UE oil. Mycelium density of *A. ustus* was intense on crude oil plates, decrease slightly on diesel and weak with UE oil while the sporulation capacity was maintained in control as in all contaminated plates (Fig. 1). Species belonging to the genus *Aspergillus* have been previously described as tolerant to hydrocarbon pollutants, *A. flavus* and *A. versicolor* were found resistant to crude oil contamination within the range of 1% to 5% (*v*/*w*) in Minimal Salt Agar and PDA medium [53]. *A. terrus* CCS2B was able to grow and tolerate up to 1000 mg of PAH mixture l$^{-1}$ in surface culture [20]. *Aspergillus* sp. showed the highest oil removal ability when cultured in PDA media plates containing 2%, 4%, 6% and until 15% (*v*/v) of crude oil [54].

The growth rate of *P. lilacinum* was significantly affected in presence of high concentration (6%) of all studied oils. The growth rates in

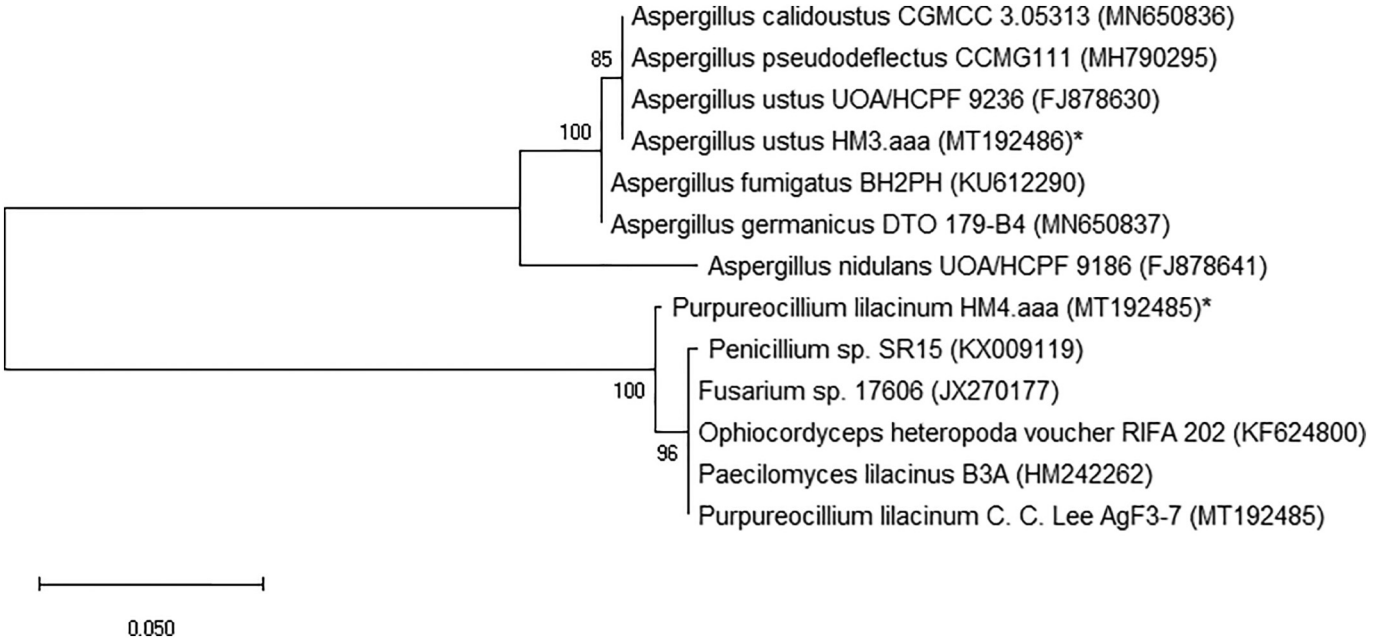


**Fig. 1.** Phylogenetic tree of the fungal isolates. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura 3-parameter model. Evolutionary analyses were conducted in MEGA X.

**Table 3**
Radial growth rate (cm $d^{-1}$) of fungal isolates in plates containing different concentrations of crude oil, diesel or UE oil. All values are means ± SD for duplicate cultures.

| Isolate | Control | Crude oil | | | Diesel | | | UE oil | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0% | 2% | 4% | 6% | 2% | 4% | 6% | 2% | 4% | 6% |
| *A.ustus* | 0.743 ± 0.004 | 0.94* ± 0.21 | 0.879 ± 0.05 | 0.770 ± 0.090 | 0,8 ± 0.007 | 0.826 ± 0.02 | 0.823 ± 0.007 | 0,83 ± 0.014 | 0.856 ± 0.118 | 0.755 ± 0.007 |
| *P. lilacinum* | 0, 438 ± 0.012 | 0.380 ± 0.045 | 0.390 ± 0.066 | 0.327* ±0.016 | 0.373 ± 0.013 | 0.281* ± 0.002 | 0.328* ± 0.024 | 0,322* ± 0.035 | 0.400 ± 0.109 | 0.315* ± 0.026 |

* Statistically significant differences ($p \leq 0.05$) in radial growth rate at different concentrations with respect to the control (0%).

contaminated plates were slower than control plate for all concentrations tested. The fastest growth rates of this isolate were recorded in the presence of 4% UE oil and 4% crude oil, respectively. A drastic reduction in growth rate (0.281 cm $day^{-1}$) was observed at 4% of diesel which decreased by approximately 36%. This behavior could be related to the effect of toxic compound in petroleum oils on fungal growth metabolism, as the toxicity of the compound is a factor that influences xenobiotic degradation process [29]. Although the growth rates on hydrocarbons were less than the controls, the macroscopic observation showed that *P. lilacinum* colony was thick in the presence of crude oil and diesel and thin in control plates (Fig. 2). It could be assumed, therefore, that *P. lilacinum* has some kind of tolerance capacity toward petroleum hydrocarbons.

### 3.5. TPHs removal assessment

For a better understanding of petroleum oils degradation ability of the selected fungi, the extraction of residual TPH from liquid culture after 40 days incubation and gravimetric analysis of the removal percent was done (Fig. 3). *P. lilacinum* exhibited the highest level of crude oil and diesel biodegradation, degrading 44.55% and 27.66% respectively. There was no significant difference between the fungal isolates in degrading diesel and UE oil. Significant difference ($p = 0.004$) was observed between *P. lilacinum* (44.55%) and *A. ustus* (30.43%) in crude oil degradation which was the most degraded oil compared to diesel and UE oil.

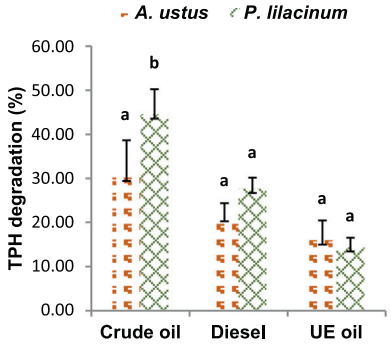


**Fig. 3.** Petroleum oils degradation rates by the two fungal strains after 40 days of incubation in mineral medium. Error bars represent standard deviations of means ($n = 3$). Different uppercase letters designate significant differences ($p \leq 0.05$) between isolates within single petroleum oil. Different lowercase letter show significant differences ($p \leq 0.05$) among petroleum oils within the same isolate.

UE oil was the least utilized hydrocarbon by the two fungi, suggesting their low capacity to degrade it. Crude oil and diesel supported the growth of the two isolates better than UE oil; this may be attributed to the fact that UE oil contains heavy metals and toxic polycyclic aromatic hydrocarbons which are highly recalcitrant [55]. Hock et al. [56] reported

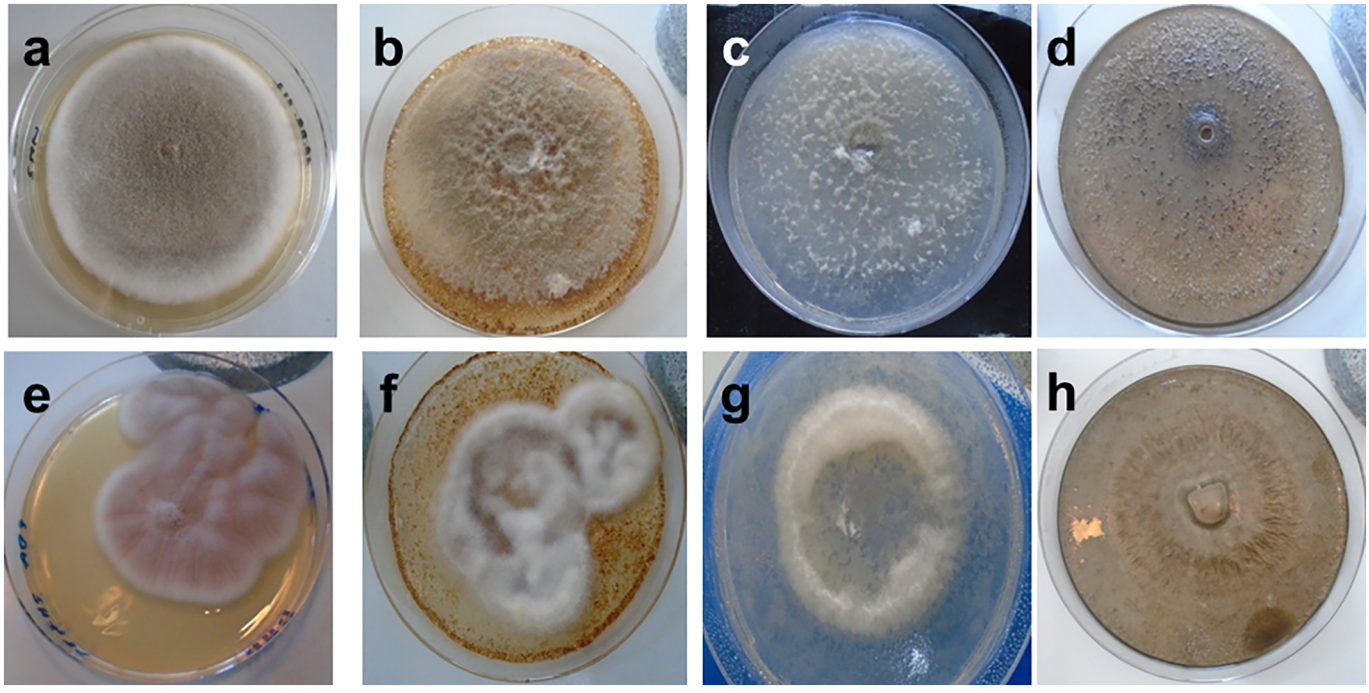


**Fig. 2.** The colony morphology of *A. ustus* on (a) PDA, (b) 6% crude oil, (c) 4% diesel and (d) 6% UE oil. The colony morphology of *P. lilacinum* on (e) PDA, (f) 6% crude oil, (g) 4% diesel and (h) 6% UE oil.

**Table 4**
Biomass accumulation by fungal isolate after 40 days incubation in mineral medium. All values are means ± SD for triplicate cultures.

| Fungal isolate | control (g/l) | Crude oil (g/l) | diesel (g/l) | UE oil (g/l) |
|---|---|---|---|---|
| *A.ustus* | 0,029 ± 0,0063$^{A,d}$ | 0,516 ± 0,011$^{A,b}$ | 1770 ± 0,169$^{A,a}$ | 0,268 ± 0,028$^{A,c}$ |
| *P.lilacinum* | 0,036 ± 0,010$^{A,b}$ | 0,419 ± 0,085$^{A,a}$ | 0,111 ± 0,024$^{B,b}$ | 0,153 ± 0,090$^{A,b}$ |

Different uppercase letters designate significant differences ($p \leq 0.05$) between means within the same column. Different lowercase letter show significant differences ($p \leq 0.05$) between means within the same row.

an *A. ustus* isolate that degrade 15.02% of UE oil in BH medium with 20% UE oil initial content, after only 7 days incubation. These data are somewhat not consistent with those noted for the same species in this study. This difference in UE oil removal ability could be related to oil composition differences, since the chemical composition of lubricating oils varies with the crude oil source, the refining process and the additives present. It depends also on the fuel type, motor operating time and the mechanical condition of the engine [57]. Nevertheless, fungi were previously reported as effective in UE oil biodegradation [14,58,59].

In the present study, Crude oil was the most degraded oil with the selected isolates; this could be attributed to the complex chemical composition of crude oil which contains different type of hydrocarbons, mostly alkanes and aromatics that are very sensitive to microbial attack [60,61]. It was equally reported that crude oil was the most successful in isolating multiple fungal genera when utilized as growth substrate [62]. *P. lilacinum* crude oil removal rate was comparable to that of Shetaia et al. [63], in which 58.15% of the total petroleum hydrocarbons in BX-crude oil were degraded by *Paecilomyces variotii* strain. In an earlier study, *Paecilomyces* sp. was found capable of mineralizing 30% of the n-alkane hexadecane in the presence of crude oil [64]. In another study, *P. variotii* showed extensive biodegradation rate of n-alkanes (>90%) present in BAL 150 crude oil [8]. Strains of the genus *Aspergillus* have been previously demonstrated to degrade crude oil in liquid medium, the biodegradation percentage of TPHs in crude oil by four active strains of *Aspergillus* genus was ≥15% [65]; *A. niger* and *A. oryzae* showed the highest level of crude-oil biodegradation ability in BH medium, degrading 54% and 99% of oil, respectively [66]. On the other side, diesel oil, which is one of the major products of crude oil, was reported to be strongly degraded with the filamentous fungus *Cladosporium*, in liquid medium, with a degradation rate of up to 34% [67]. *A. fumigatus*, isolated from diesel oil storage tank sludge, was very efficient in degradation of diesel oil, especially the aliphatic fractions [68]. Similarly, two fungi of the genus *Geomyces* were found to be effective in diesel fuel biodegradation, in 1% oil supplemented mineral broth, the rates were 77.3% and 68.6% [16].

### 3.6. Fungal biomass assessment after biodegradation

The fungal dry biomass of the culture broth after biodegradation was measured (Table 4). The two isolates showed greater accumulation of biomass in presence of petroleum hydrocarbons as compared to their corresponding controls, suggesting the ability of *P. lilacinum* and *A. ustus* to use these oils as a carbon source. *A. ustus* has shown the highest biomass accumulation for all petroleum hydrocarbons when compared with *P. lilacinum*. Ramoutar et al. [69] established that *Aspergillus* species were more active than other species toward hydrocarbons, producing the highest dry biomass. In our study, significant differences were found in the amount of biomass produced by *A. ustus* on crude oil, diesel, UE oil and the control. The highest amount of dry biomass was recorded by *A. ustus* in diesel (1.770 g/l) followed by the crude oil (0.516 g/l). Biomass production of *P. lilacinum* showed no significant differences between diesel, UE oil and the control. However, significant differences were found between crude oil and all other hydrocarbons and the control. *P. lilacinum* produced the highest dry mass with crude oil while the lowest amount of biomass where observed with diesel and UE oil. Most fungi that utilize petroleum hydrocarbons as a source of carbon and energy metabolized the molecules to $CO_2$ and biomass [70]. Fungi showed different behavior in utilization of each petroleum oil as source of energy and/or biomass. The chemical composition of each oil is an important factor that determine the ability of fungal isolate to grow on it, this assertion is further supported by the observation of Davies and Westlake [34], where the growth of individual fungal isolates on seven crude oils of varying composition was not always similar even on oils which have essentially similar n-alkane profile. Fungal biomass production on diesel have been previously reported for *Aspergillus* species, *A. terrus* showed greater accumulation of biomass (0.831 g/l) during degradation of diesel as compared to corresponding control [37]. *A. fumigatus* reached the highest values of biomass weight (approx. 60 mg) after 60 days incubation in mineral medium with only diesel as carbon source [68]. *P. lilacinum* has previously showed the greatest increase of biomass in the presence of hexadecane and toluene [48]. Ameen et al. [37] reported 30.9% gain in weight biomass of *P. variotii* via biodegradation of diesel in liquid medium.

### 3.7. Crude oil biodegradation kinetics

Crude oil was the most degraded oil by the two selected fungi. Therefore, the biodegradation of this oil during time was evaluated every 10 day interval for a total of 40 days in liquid medium (Fig. 4). Biodegradation rate constant (k) and half-life time ($t_{1/2}$) were determined using first-order kinetics model and presented in Table 5. Progressive decrease in crude oil and concurrent biomass increase were observed in liquid media with both isolates. There was a rapid reduction in crude oil concentration within the first 20 days of the study. At the end of the 20 days, there was 34% and 28% TPH reduction in media with *P. lilacinum* and *A. ustus*, respectively. TPH removal kinetics showed that *P. lilacinum* removed crude oil with the highest rate 0,02 $day^{-1}$, similarly, the half-life which is the time required for removal of the half TPH initial concentration was shorter in *P. lilacinum* ($t_{1/2}$ = 34,66 days) than in *A. ustus* ($t_{1/2}$ = 46,21 days). The data showed that fungal isolates grew as they degraded the crude oil; the same results were reported previously for the fungus *Trematophoma* sp. which

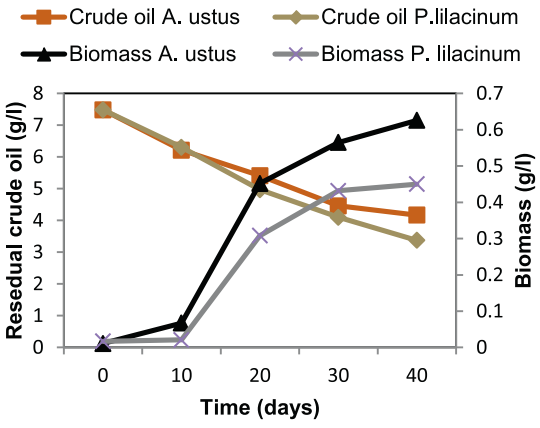


**Fig. 4.** Crude oil degradation and biomass accumulation by fungal isolate during 40 days incubation in mineral medium.

**Table 5**
Parameters of the first-order biodegradation kinetics of crude oil by fungal isolate in mineral medium.

| Isolate | $k\ (day^{-1})$ | $t_{1/2}\ (day)$ | $r^2$ |
|---|---|---|---|
| *A.ustus* | 0.015 | 46.21 | 0.982 |
| *P.lilacinum* | 0.02 | 34.66 | 0.998 |

revealed a progressive increase in crude oil degradation and biomass production in mineral broth during 15 days of incubation [21]. The value of the kinetic parameter showed effectiveness of the two isolates in biodegradation of crude oil, the half-life values obtained in this study were in line with those reported by Essabri et al. [71] for biodegradation of TPH by *A. niger* and *Penicillium ochrochloron* within a period of 60 days.

## 4. Conclusion

The study of filamentous fungi for bioremediation applications exposes their potentials especially for contaminants with limited access to microorganisms capable in their degradation. The current study showed successful degradation of crude oil, diesel and UE oil in liquid culture by two filamentous fungi *A. ustus* and *P. lilacinum* isolated from an artificially contaminated soil. The two isolates displayed good crude oil and diesel degradation ability, crude oil was the most degraded oil. In addition, the results of this research show that *P. lilacinum* was more potent in hydrocarbons degradation and exhibited the highest crude oil (44.55%) and diesel (27.66%) removal rates, the highest biodegradation constant (0,02 $day^{-1}$) and the shortest half-life ($t_{1/2}$ = 34,66 days) period for crude oil biodegradation. This study provides enough information on the ability of *P. lilacinum* to degrade three petroleum oils in liquid culture. However, supplementary studies are still needed to investigate the application of this fungus for successful field-level bioremediation processes. Further studies are suggested to examine the potential of candidate fungi for hydrocarbons degradation in soil microcosms and ecotoxicity tests must be performed to evaluate the effectiveness of bioremediation approach for soil preservation and rehabilitation.

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## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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