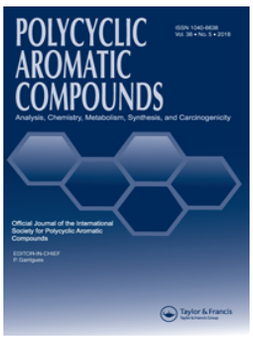

Polycyclic Aromatic Compounds

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Pyrene-Degrading Fungus *Ceriporia lacerata* RF-7 from Contaminated Soil in Iraq

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs), such as pyrene (PYR), are toxic to the environment. Bioremediation is considered a safe and economically technical approach to remove PAH from the environment. Fungi can grow on several hydrocarbons, including PYR. This study aimed to isolate and identify PYR-degrading fungus from the contaminated soil of Rumaila oil-field. Strain RF-7 was classified as a member of the *Ceriporia* genus on the basis of the internal transcribed spacer sequencing and the morphological properties. The growth of RF-7 at different concentrations was investigated using the microtiter plate method. The total PYR degradation by RF-7 was quantified using gas chromatography. With 20 mg/L PYR, 55.5% PYR was degraded after eight days of incubation. The co-substrate (glucose) experiment revealed that the PYR degradation efficiency of strain RF-7 can be substantially improved. After eight days of incubation, 90.5% of PYR (with a co-metabolism substrate) was degraded by RF7. Based on the above findings, the strain RF-7 can degrade PYR effectively and may be applied in remediating PAH-containing soils.

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Biodegradation; cometabolism substrate; fungi; Pyrene

Introduction

Polycyclic aromatic hydrocarbons (PAH) are a class of environmental pollutants. Many PAHs have mutations/carcinogens and disrupt the activity of the endocrine system.[1] PAH, one of the main components in crude and heavy oils, is easily formed by the incomplete combustion of organic compounds, such as fossil fuels.[1,2] Oil refining and transport activities are the major contributors to the localized pollutants of PAHs into the environment. These pollutants can occur through the industrial effluent discharge, raw accidental release, and products of refining.[3] Pyrene (PYR), a PAH comprising four fused benzene rings, commonly exists as a pollutant of soil, water, and air.[4,5] PAH has high resistance to biodegradation and is persistent in the environment.[6] PYR is briefly nitrated to form nitropyrene, which is highly carcinogenic. However, no evidence exists that PYR is carcinogenic to humans and nonhumans.[4] These concerns necessitate the urgent need to study the mechanisms of PYR degradation by active microbial process before it is

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converted into a carcinogenic compound.[6,7]Numerous microbial degradation studies have reported that removing high-molecular weight PAHs with four and/or more fused aromatic rings is harder than removing low-molecular weight PAHs.[5,8] In this study, PYR was used as a model compound to determine the biodegradation of high-molecular weight PAHs due to its similarity in structure with several carcinogenic PAHs.[8,9] Many bacterial isolates have been shown to grow on or during PYR degradation,[10–13] but relatively few studies have used fungi.[14,15] The *Coriolopsis byrsina* strain APC5 has shown a high level of PYR degradation.[9] Romero, Salvioli, Cazau, Arambarri[14] have proven the metabolism of the four-ring PAHs by filamentous fungi. *Fusarium solani* can highly oxidize PYR. Three fungal strains are also isolated from a soil in Japan, and they assimilate PYR as the sole source of carbon and exhibits high PYR degradation activity.[16] Marine-derived fungi (*Tolypocladium* sp. CBMAI 1346) can degrade up to 95% PYR within seven days of incubatio.[17] Hadibarata, Kristanti[18] have observed that the white-rot fungus, *Armillaria* sp. F022, exerts great ability for PYR degradation. Zafra, Cortés-Espinosa[19] reported that the *Trichoderma* species have a high ability to degrade PAHs such as PYR, and it can be used as potential bioremediation agents in soils contaminated with petroleum hydrocarbons. These studies suggest that fungi promote the active transport and spatial dispersion of PAH.[13,20] Several contaminated sites are polluted with several materials. Thus, PAH-degrading bacteria and fungi are necessary to efficiently remediate contaminated sites. Few studies have examined the abilities of fungi to degrade high-molecular weight PAHs. In this study, the isolation, growth, and some properties of the fungus with the ability to utilize PYR are described. The effects of adding glucose to the PYR medium with *Ceriporia lacerata* RF-7 are also investigated.

Materials and methods

Sample collection and fungal isolation

Soils were collected from the Rumaila oilfield, Basra, Iraq, and the fungus was isolated from contaminated soil by the following methods. Sterile water (9 mL) was mixed with contaminated soil (1 g), and the mixture was vigorously shaken and diluted stepwise with sterile water. The mixture (1 mL) was inoculated in 10 mL mineral salt media (MSM; composed of gram per liter deionized water, KH_2PO_4 0.4, $K_2HPO_4{\cdot}3H_2O$ 1.0, NaCl 0.5, $NaNO_3$ 0.2, $(NH4)_2SO_4$ 0.1, and $MgSO_4{\cdot}7H_2O$ 0.025) with 1% crude oil and 50 mg/L chloramphenicol in test tubes at 30 °C and 120 rpm. An enrichment culture was inoculated on the MSM. The enrichment culture was diluted in MSM, spread on the MSM–PYR agar plate, and incubated at 30 °C. The acquired single colonies were transferred on the MSM–PYR plate. The single colonies were inoculated again on the MSM–PYR, and the isolated fungus was preserved on an agar slant.

The fungal spores were isolated through inoculation on potato dextrose agar (PDA) plates at 30 °C. The generated spores on the plates were harvested, suspended in sterile water, and adjusted to an absorbance of 0.6 at 600 nm. During the degradation experiment, the spore suspension was inoculated in MSM supplemented with 20 mg/L PYR at 30 °C and 150 rpm for eight days. PYR (1 g/L) was used in the medium to study the effect of cosubstrates (glucose) in the degradation of PYR.

DNA and molecular extraction

The genomic DNA of the isolated fungus was extracted using the mycelium CTAB method described by Doyle.[21] The fragments of DNA were amplified using the T100 Thermal Cycler (Bio-Rad, California, USA). An internal transcribed spacer (ITS) region was amplified using the forward primer 5′-TCCGTAGGTGAACCTGCGG-3′ and the reverse primer 5′-TCCTCCGCTTATTGATATGC-3′. For sequencing, the 3730xl DNA sequencer (ABI, USA)

Tsingke (Tsingke Biological Technology, Co., Ltd.) was used. A homology search was carried out using the BLAST in the NCBI website (http://www.ncbi.nlm.nih.gov). The phylogenetic tree was constructed using the neighbor-joining method and the MEGA Software (version 6.0).

Measurement of PYR

Pyrene (PYR: purity $\geq$ 97% chemical formula $C_{16}H_{10}$; molar mass: 202.25 g/mol; melting point: 145 °C–148 °C; 293 °F–298 °F, 418–421 K; boiling point: 404 °C, 759 °F, 677 K) was purchased from Aladdin Chemistry Co., Ltd. The stock solution of PYR (2 g/L in cyclohexane) was prepared in advance. The remaining PYR was extracted twice with 10 mL cyclohexane, and a layer of cyclohexane was dehydrated with anhydrous sodium sulfate, concentrated using a rotary evaporator, and exposed to a nitrogen stream. Finally, PYR was dissolved in 1 mL cyclohexane and analyzed using the external standard method by employing the gas chromatography MIDI-Sherlock (Agilent, USA) equipped with a flame ionization detector. PYR was separated on the 19091z-433 PH-1 capillary column (30 m $\times$ 0.25 mm $\times$ 0.25 µm, 35 °C; carrier: Nitrogen, 42.1 mL/min).[22] PYR depletion was calculated using the formula as described in our previous study.[23]

Microtiter assay for growth of Ceriporia sp. RF-7

The effect of PYR concentrations (5, 10, 15, 20, 25, and 30 mg/L) on the growth of RF-7 spore was investigated at 30 °C for eight days. The experiment was carried out using 96-well microtiter plates, which contained MSM supplemented with PYR. The control was maintained under the same conditions without PYR for comparison of results. The fungal spores of strain RF-7 were isolated from the 7-day old PDA flask by using the method described in our previous study.[24] The growth of strain RF-7 was estimated using the Langvad method, and the optical density at 630 nm was determined using a microplate reader (Thermo Fisher, Waltham, Massachusetts, USA).[25] The inhibition of growth was measured by comparing the growth of RF-7 with that of the control.

Liquid culture experiment for PYR biodegradation at different conditions

The liquid culture study was conducted to determine the degradation efficiency of the strain RF-7. The strain RF-7 was inoculated in 50 mL Erlenmeyer flasks containing 10 mL MSM supplemented with 20 mg/L PYR. The spore suspension (100 µL) was inoculated into sterile MSM. The flasks were incubated at 30 °C for eight days at an interval of two days. The effects of pH and temperature on the degradation of PYR by strain RF-7 were examined over a pH range of 2.0–8.0 at 30 °C and at an incubation temperature range of 10 °C–40 °C at pH 7.0. Glucose (1 g/L) was added to the medium to study the effect of cosubstrate on the degradation of PYR. All tests were carried out in three independent replicates.

Statistical analysis

Each experiment was conducted in triplicate. All data generated during the study were subjected to one-way ANOVA and least significant difference test using the SPSS version 18.0. The percentage of PYR degradation (D%) was calculated by the formula: $D\% = 100(IC - FC)/IC$, where IC is the initial concentration of the PYR, and FC is the concentration at the sampling time.

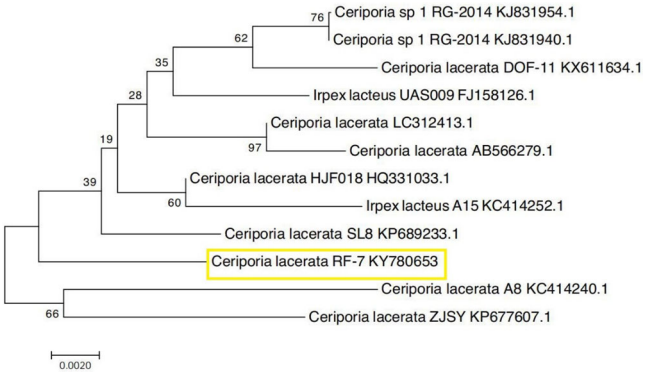

Figure 1. Phylogenetic tree of strain RF-7.

Results and discussion

Isolation and genetics of PYR-degrading fungus

The PYR-degrading fungal strain was obtained from soil samples in Rumaila oilfield, Basra Iraq. The DNA of the isolated strain was purified, and the fragments of the ITS region were amplified by polymerase chain reaction and sequenced. The measured sequence of the fragments was from 659 bp (accession number: KY780653). From the result of a homology search on BLAST, the isolated fungus was found to belong to the *Ceriporia* genus. The phylogenic tree of *C. lacerata* RF-7 is illustrated in Figure 1.

Microtiter assay of Ceriporia sp. RF-7 growth

The growth of RF-7 against different concentrations of PYR was revealed using the microtiter experiment (Figure 2). Results revealed significant differences in the growth of RF-7 at different concentrations of PYR. The growth of strain RF-7 increased when the PYR concentration in MSM ranged from 5 mg/L to 20 mg/L after eight days of incubation and was significantly lower when the PYR concentration in MSM was $\geq$ 25 mg/L. The diversified concentrations of substrate exerted different effects on the RF-7 growth. When the PYR concentration increased, the growth of strain RF-7 declined, which may be due to the toxicity of PYR at high concentrations.[23,26] Our results showed that the RF-7 growth declined when PYR was higher than the suitable concentration, which was likely because of the various toxicities. High substrate concentrations are toxic to the microorganism, whereas low substrate concentrations may not provide enough carbon source for the growth of the microorganism. Thus, exploring the ability of microorganisms to adapt to the environment with various concentrations of substrate is necessary. The growth of RF-7 increased with time during the eight days of incubation, demonstrating that the RF-7 utilized PYR as sole carbon and energy source for growth. Bacteria and fungi can use PAHs as sole carbon sources.[27,28]

Effect of pH on PYR degradation

The appropriate pH can play a significant role in biological methods, and the effects of pH and temperature on PYR degradation are illustrated in Figures 3a,b. During incubation, the strain RF-7 exhibited that it can grow over a wide range of pH. The degradation percentages of DBA in the presence of the strain RF-7 were 20.2%, 38.3%, 44.3%, 55.5%, and 40.2% at pH 2, 5, 6, 7, and 8, respectively, on day 8. This result indicated that the RF-7 exhibited good PYR degradation at neutral or weak alkaline condition and low PYR degradation under acidic condition. The highest

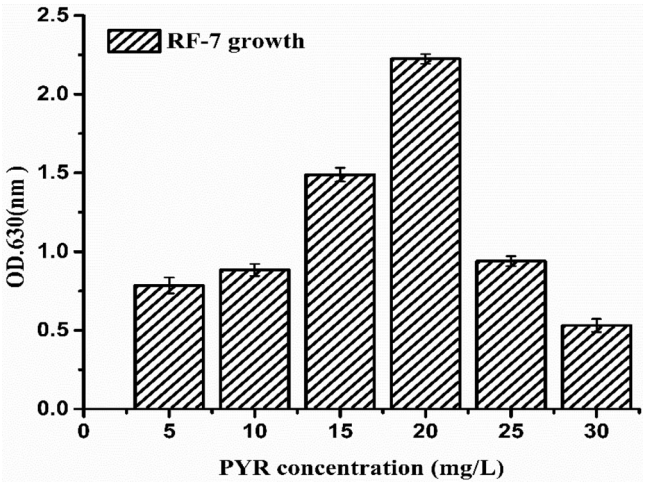

Figure 2. Fungal growth (as O.D. 630 nm) of strain RF-7 in various concentrations of PYR (5-30), all the values given were the averages of triplicate independent experiments. Error bars represented the S.D.

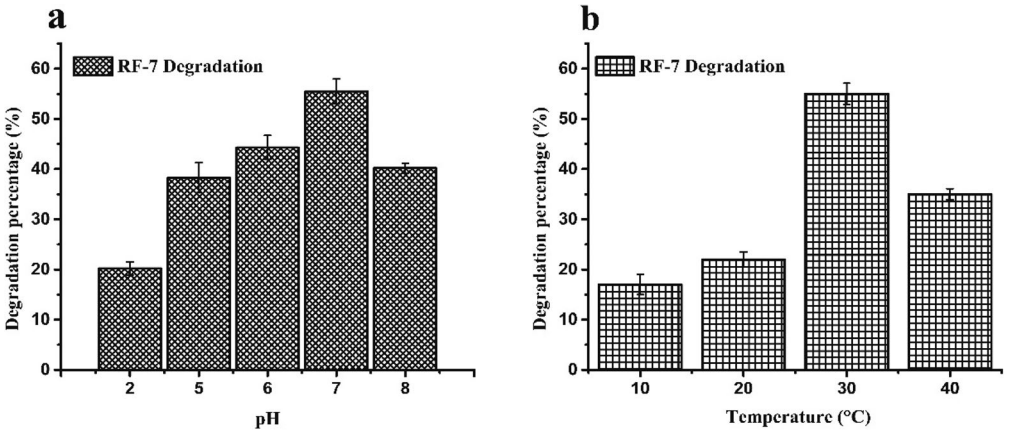

Figure 3. Degradation percentage of PYR under different pH (a) and temperature (b). All the values given were the averages of triplicate independent experiments. Error bars represented the S.D.

PYR degradation percentage occurred in cultures with initial pH of 7.0, which was considered the most suitable condition in the degradation experiment. Thus, the optimum pH value of the cultures was 7.0. Microbial activity was greatly affected by pH, and microbial growth and metabolism were closely related to pH. Several studies have reported that the best pH for PAH degradation is not the same for different microorganisms.[29] The best pH is slightly alkaline for some microbes and acidic for others.[23,29,30] This conclusion was similar to that obtained in the current study. The appropriate growth pH of strain RF-7 was neutral or weakly alkaline. The findings of the degradation experiment also showed that the suitable pH for PYR degradation by strain RF-7 was 7.0. Our findings were compatible with the previous report of Mineki, Suzuki, Iwata, Nakajima, Goto[16] on PYR degradation by fungi. Temperature is one of the significant factors that affects petroleum degradation due to its effect on the natural chemical and physical compositions of the petroleum.[29] Biodegradation increased with temperature ranging from 10 °C to 40 °C, and the optimal temperature for biodegradation was 30 °C. The increase in degradation at a specific temperature range may be due to the increased energy efficiency in the system.[29] High temperatures, naturally in the range of 30 °C to 40 °C, increased the hydrocarbon

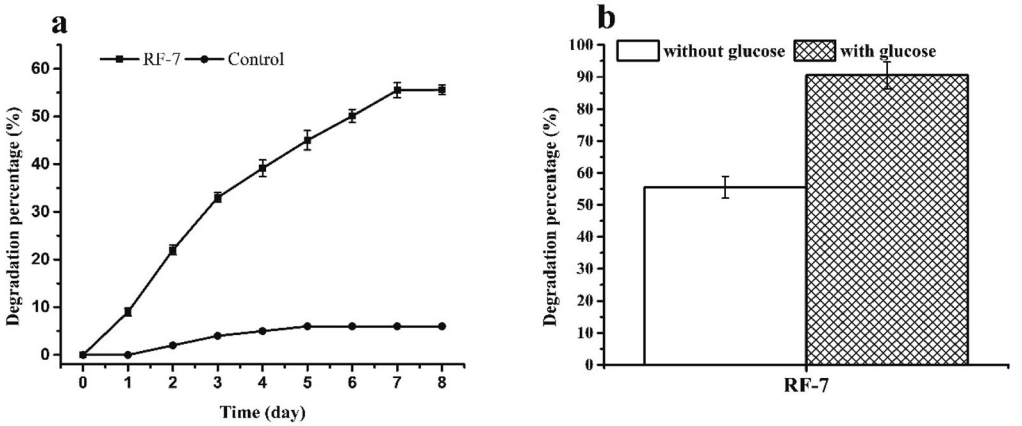

Figure 4. (a) Degradation percentage of PYR (20 mg/L) by strain RF-7 grown for 8 days at 30 °C and 7.0 pH. Degradation percentage of PYR of strain RF-7 in MSM media with/without glucose (as co-substrate). All the values given were the averages of triplicate independent experiments. Error bars represented the S.D.

metabolism rates to a maximum. No significant change in the degradation of PYR by the strain RF-7 was noted within the investigated temperature range (30 °C–40 °C).

PYR degradation and the effect of glucose on PYR degradation

The PYR concentration in the MSM after different incubation periods is illustrated in Figure 4a. The degradation of PYR increased with incubation time and increased speedily until day 7 and then increased slowly. After eight days of incubation and using 20 m/L PYR as a sole carbon source, 55.5% PYR was degraded. The decrease in PYR concomitantly occurred with the increase in fungal growth. The degradation percentage of PYR was highest at day 7 and stabilized on day 8. Results revealed that the PYR concentration was significantly reduced within two days of incubation compared with the control. The PYR content was found to be minimum (8.9 mg/L) against its initial value and control (18.8 mg/L). The residual concentration of PYR in the control (without RF-7) showed no significant change within eight days. Some fungi can withstand and degrade the aliphatic and PAHs in water and contaminated soil.[23,31–33]

Mineki, Suzuki, Iwata, Nakajima, Goto[16] have proven that the fungal strains isolated from the contaminated soils in Japan can degrade pyrene for use as sole carbon source. Saraswathy, Hallberg[34] have reported that the fungi isolated from the soil of a former gaswork site can degrade PYR. *Armillaria* sp. *F022,* a white rot fungus, uses PAH as sources of carbon and energy, and 63% PYR has been degraded by *Armillaria* sp. F022 during incubation in a liquid media.[18] The *Coriolopsis byrsina* strain APC5 is isolated from Chhattisgarh and has a high capacity to degrade PYR, which is estimated to be 96.1% with the aid of their ligninolytic activity.[9] *Scopulariopsis brevicaulis* can degrade 64% PYR in liquid culture.[35] In this study, *C. lacerata* RF-7 was reported to degrade PAH (PYR) for the first time. Wang, Chu, Wu, Zhao, Xu,[36] Lin, He, Han, Tian, Hu,[37] and Xu, Zhu, He, Han, Tian[38] have reported that *C. lacerata*, *C. lacerata* P2, and *C. lacerata strain* P2 fungi, respectively, can degrade dye, but no report is available on the PAH degradation.

For the remediation of PYR, the effect of glucose added to the MSM was investigated in Figure 4b. The source of carbon in the medium is a significant factor for the growth and metabolism of a microorganism. Many studies have proven that saccharides can stimulate the degradation of PAHs by fungi.[39,40] Previous studies have observed glucose as the best carbon source (cosubstrate) for the degradation of PAHs by fungal strains.[40,41] Thus, glucose was selected as the carbon source (cosubstrate) for PYR degradation, and the effect of glucose (1 g/L) was examined.

Results showed that the use of 1 g/L glucose led to 90.5% PYR degradation and that the absence of glucose led to a lower level of PYR degradation (55.5%) than with glucose in the MSM. These results revealed that the use of 1 g/L glucose resulted in the highest degradation of PYR. Govarthanan, Fuzisawa, Hosogai, Chang[40] found that the cultures enhanced the degradation activity of *Penicillium* sp. CHY-2 at 1 g/L glucose. Ron, Rosenberg[42] reported that using glucose as the cocarbon source resulted in higher levels of *n*-eicosane degradation compared with the control. Choosing an ideal and cheap carbon source accelerates the fungal growth and the bioremediation process.

Conclusion

In view of the findings obtained in this work, the strain *Ceriporia* sp. RF-7 isolated from the contaminated soil of Rumaila oilfield exhibited great ability in the degradation of PYR at 30 °C and pH 7.0. The degradation of PYR (55.5%) by RF-7 indicated its hydrocarbon degradation capability. The data presented in this work described that RF-7 had a potential to degrade PYR, and the cometabolism substrate improved the PYR degradation efficiency by 90.5%. In addition, this study represented the first report of the participation of *Ceriporia* RF-7 genera in PYR degradation. Future studies will focus in the biodegradation capabilities of the isolated microorganisms.

Acknowledgments

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Disclosure statement

Authors have no potential conflict of interest to disclose.

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