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First isolation of biodegradable polycyclic aromatic hydrocarbons *Mycobacterium porcinum* and *Mycobacterium celeriflavum* from oil-polluted ecosystems

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Abstract

Background: Polycyclic aromatic hydrocarbons (PAHs) have detrimental effects on human, ecosystem, and biodiversity. Bioremediation is an option that has been used to remediate and reduce the risk of contaminants such as PAHs. Microorganisms are readily available to screen and can be rapidly identified to be used in many extreme environmental conditions. Mycobacteria have a great potential for the production of bioactive compound, which have degradation activity. Due to this issue, and also, as there is no study conducted on the biodiversity of biodegradable *Mycobacterium* in Markazi province, the present study aimed to assess the isolation and identification of biodegradable *Mycobacterium* species from diverse Markazi province ecosystems.

Methods: *Mycobacterium* were screened from a total of 30 soil, water, and sludge samples from the oil-polluted ecosystems of Markazi province and characterized to the genus and species level by applying molecular and conventional microbiological assay including the PCR amplification and sequence analysis of 16SrRNA and hsp65 genes. The growth rate in the presence of PAHs, turbidometry, and high performance liquid chromatography (HPLC) were used to determine their bioremediation capability.

Results: In total, 6 *Mycobacterium* isolates (20%) were screened from 30 samples, which belonged to two species of *Mycobacterium* consisting of *M. porcinum* (4 isolates) and *M. celeriflavum* (2 isolates). The strains of *M. porcinum* and *M. celeriflavum* could degrade 70% and 90% of 1 mg/L PAH solution in 7 days.

Conclusion: According to the results, the *M. porcinum* and *M. celeriflavum* have a significant capability to biodegrade the PAHs. Therefore, more investigations are recommended for separation and applicational use of the mycobacterium species for bioremediation of PAHs.

Keywords: 16SrRNA, Biodegradation, Nontuberculous *Mycobacterium*, Chromatography

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) are crude oil derivatives that consisted of two or more benzene rings and are one of the most important constituents of crude oil. These compounds are often present as complex mixtures in nature and are not seen individually. PAHs purely are colorless or yellow solids used in paint, plastics, pesticides, and asphalt production (1).

These compounds in humans can lead to the diseases such as cancers (2, 3), reproductive problems (4), respiratory diseases (5, 6), skin damage, hearing problems and neurological diseases (7), as well as functional disorderliness or destruction of the endocrine organs and liver because of toxicity (8). Moreover, by contaminating the water and soil resources, such compounds eventually destroy the ecosystem (9). These disadvantages have caused PAHs-containing compounds to be considered as one of the most important environmental pollutants. Therefore, purification and decontamination of contaminated areas is of great importance.

In recent decades, numerous physical and chemical assay such as incineration and dredging, light oxidation, evaporation, adsorption, chemical oxidation, and leaching of soil particles have been applied to degrade and recycle pollutant (10, 11). Hence, these assays are not cost-effective and ecofriendly and due to producing and emitting secondary pollutants may cause further damages to the environment (12). Bioremediation based

on safety, ecofriendly, and low cost is an effective method for remediation and neutralization of environmental pollutants. Bioremediation is a process that uses organisms, mostly plants, and microorganisms to breakdown, reduce toxicity or detoxify pollutants and waste products. Therefore, the most important step in bioremediation process is the isolation and identification of organism that have biodegradable potential of a particular or a group of pollutants (13).

Microflora develops in the regions where they can survive in the presence of such undigested materials and can use these compounds as an energy and carbon sources and they grow and appear as the predominant microbial population of the area (14). Several studies conducted in this field showed that numerous aquatic and soil bacterial species can degrade and consume pollutants as a sole carbon and energy source, such as *Polaromonas*, *Pseudomonas*, *Burkholderia*, *Bacillus*, *Sphingomonas*, *Nocardia*, and *Mycobacterium* (15, 16). *Mycobacterium* genera are reported to not only have a high intrinsic resistance to stressful condition but also have the potential for degradation of environmental pollutants including crude oil, hydrocarbons, chlorophenols, polychlorinated biphenyls polychlorophenols, heavy metals, and diverse PAH alkanes (17). Moreover, *Mycobacterium* has a slower growth rate compared with other bioremedial microorganism, therefore, it can survive in unfavorable conditions in contaminated environment. Although, they can successfully compete with fast growing genera such as *Bacillus* and *Pseudomonas* that are well-known for their capability in degradation of pollutants like PAHs (18,19). Therefore, by using family members of actinomycetes in the biodegradation process, a suitable strategy for neutralizing PAH compounds from contaminated environments can be provided.

Based on the high biodiversity of microorganism with unknown bioactive ability in Iranian ecosystems, and high biodegradation potential of *Mycobacterium* genus, the aim of this study was to screen and identify *Mycobacterium* with bioremediation potential from diverse environmental samples of Markazi province ecosystems.

Materials and Methods

From May 2018 to July 2019, a total number of 30 water, river and lake sediments, sewage of urban, hospital, barracks, industries, and livestock samples of various environmental resources of Markazi province ecosystems were collected (Figure 1).

The collected samples were analyzed according to standard methods that have been applied in the previous study (20). In brief, collected water samples were exposed to cetylpyridinium chloride 0.005 for 15 minutes to reduce the contaminant microorganism. They were then filtered with 0.45 µm membrane filters (Cellulose nitrate, Sartorius AG, Germany). Then, filters were transferred into tubes containing 20 ml of sterilized water, and were

Figure 1. Geographic distribution of sampling site from Markazi province ecosystems. The figure source is from Iran National Mapping Agency and designed by Adobe Photoshop 2020 v 21.2.2.289.

mashed and soaked. At the end, 100 µL of the soluble specimens were transferred to the Lowenstein-Jensen (LJ) and Sothon media and incubated at temperatures of 20, 30, and 35°C and in the presence of 5-10% CO_2.

In the case of soil sampling, 5 g of soil sample was added to the sterile tube contained 15 mL of sterilized water, and they were centrifuged at 4000 × g for 10 minutes. The supernatant was moved to a new tube and sterilized by adding NaOH 3% and sodium lauryl sulfate1%. Then, 100 µL of sterilized samples were used for inoculation into the Sothon and LJ media, then, incubated as mentioned above (21).

For analysis of sediment samples, 3 g of samples were mixed in 50 mL of 8% normal saline for 30 minutes, and a uniform suspension was prepared. Then, suspensions were diluted 1/10 in distilled water and 200 µL of each dilution of 10-2, 10-3, 10-4 was added to the satin medium containing antibiotics such as antifungal and antibacterial agents for instance nalidixic acid, Nystatin, and kanamycin (50 µg/mL each). The specimens at 20, 30, and 35°C in the presence of 5-10% CO_2 were incubated for 3 months (20, 22).

The details of analyzed samples in the present study are presented in Table 1.

Microbiological characterization of the Mycobacterium isolates

By using common phenotypic and biochemical tests, *Mycobacterium* isolates were identified to the extent of genus level. The tests were used for identification of isolates including acid-fast staining, colony morphology, and biochemical tests including heat-stable and semi-quantitative catalase production and constant temperature (68°C), semi-transparent colonies, pigmentation, nitrate reduction, and niacin consumption (23).

Molecular identification of Mycobacterium isolates

The mycobacterial DNA was extracted using the Pitcher method (16). Specific PCR amplification of a 620 bp fragment of heat-shock protein gene (*hsp65*) was used for molecular genus identification of isolates (24). In

Table 1. Samples profile, phenotypic and molecular features and bioremediation analysis of mycobacterial isolates from Markazi province ecosystems

Sample Profile			Phenotypic Features									16S rRNA Analysis				
Isolates	Location (city) province	Source	pH	Temp	OT	GR	Semi-quantitative catalase	Tween 80 hydrolysis	Tolerance of NaCl 5%	PncA	Reduction of potassium tellurite	Urease	Runyon group	Similarity (%)b	Base pair differences	Identification	Biodegradation activity
A4	Khomein (Markazi)	Land farm soil	7	14	25	R	+	+	-	-	+	+	IV	100	0/1356	*M. porcinum*	PAHs
A8	Arak (Markazi)	Petrochemical factory soil	8.3	18	30	R	+	-	+	-	-	-	IV	100	0/663	*M. celeriflavum*	PAHs
A11	Arak (Markazi)	Salt lake sediment	7	16	35	R	+	-	+	+	+	+	IV	100	0/1044	*M. celeriflavum*	PAHs
A14	Mahalat (Markazi)	Sarcheshmeh sediment	8.4	16	25	R	-	+	+	-	+	-	IV	99.9	1/1067	*M. porcinum*	PAHs/crude oil
A15	Shazand (Markazi)	Refinery	6.4	25	25	R	+	+	+	-	-	+	IV	99.9	1/1023	*M. porcinum*	PAHs/crude oil
A3	Shazand (Markazi)	River sediments	7.8	16	35	R	-	+	-	+	+	-	IV	99.9	1/1023	*M. porcinum*	PAHs/crude oil

Abbreviations: Temp, temperature; OT, optimum temperature ; GR, Growth Rate; PncA, pyrazinamide.

brief, the PCR amplification reaction with either of the primer pairs *hsp*65F 5'ACCAACGATGGTGTGTCCAT3' and *hsp*65R 5'CTTGTCGAACCGCATACCCT 3' was performed using Ex-Taq DNA polymerase and the compatible PCR reagents (KiAGEN). The reaction mixture (50 µL) consisted of 5 µL of DNA template, 200 µM of each of the four deoxynucleoside triphosphates, 1× Ex-Taq buffer with MgCl2, 100 ng of both forward and reverse primers, and 1.25 U of ex-Taq DNA polymerase. The presence of PCR products was determined by electrophoresing 10 µL of the reaction product on a 1% agarose gel matrix (Merck, Germany) with 1× Tris-EDTA buffer containing safe stain (0.5 µg/mL) and using 5 µL of a 100-bp DNA size marker. Each PCR product was further quantitated and photographed using the Kodak EDAS 290 gel documentation system (Kodak, Rochester, N.Y.) (Figure 2a). PCR amplification and direct analysis of 16SrRNA gene sequences were used for identification of species of isolates. The 16s rRNA gene was amplified using pairs of primers that yielded 1550 bp DNA fragments. The sequences of the oligonucleotide primers were as follows: 27F: 5' AGA GTT TGA TCM TGG CTC AG3' used in combination with 1492R: 5'CGG TTA CCT TGT TAC GAC TT3'. PCR amplification reaction was performed in the same procedure as mentioned above (Figure 2b) (25). The PCR product was sequenced at Bioneer Company (South Korea). Then, the received sequences were aligned with closely related *Mycobacterium* species sequences taken from the GenBank TM database, and the sequences were compared using jPHYDIT software (Figure 3) (26).

GenBank accession numbers of nucleotide sequence

The 16SrRNA sequences GenBank accession numbers of isolated *Mycobacterium* in the present study are *M. porcinum* (MK693740) and *M. celeriflavum* (MK693737).

PAHs bioremediation analysis of isolates

The biodegradation ability of *Mycobacterium* isolates was measured according to the proposed method by sun et al. as follow (27). To analyze the growth of isolates in the presence of PAHs, 150 mL of mineral salt medium (MSM) was transferred into 300 mL sterile flask as the basal medium for experiment. The MSM media was composed of K_2HPO_4 (0.375), $MgSO_4{\cdot}7\ H_2O$ (0.05), KH_2PO_4 (0.42), $(NH_4)2SO_4$ (0.244), $CaCl_2{\cdot}2H_2O$ (0.015), NaCl (0.015), and $FeCl_3$ (0.054). The MSM media with 1% PAH mix suspension (1-1) purchased from AccuStandard was supplemented. The PAHs suspension containing the following components: Naphthalene, benzo-pyrene, anthracene, fluorene, acenaphthene, indeno-pyrene, benzo-fluoranthene, acenaphthylene, 1,2-benzanthracene, chrysene, benzo-fluoranthene, benzo-perylene, fluoranthene, dibenz-anthracene, phenanthrene, and pyrene, each at a concentration of 0.2 mg/mL was dissolved in methanol and dichloromethane.

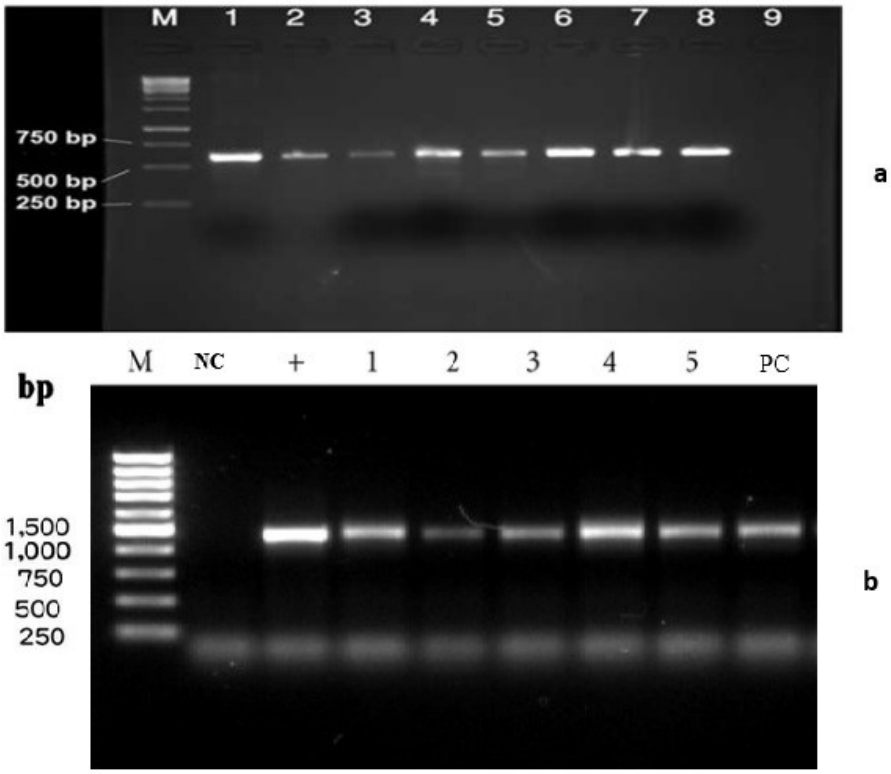

Figure 2. Gel electrophoresis of *hsp65* (a) and 16S rRNA (b) PCR products of Iranian mycobacterial isolates run on the 1% agarose gel.

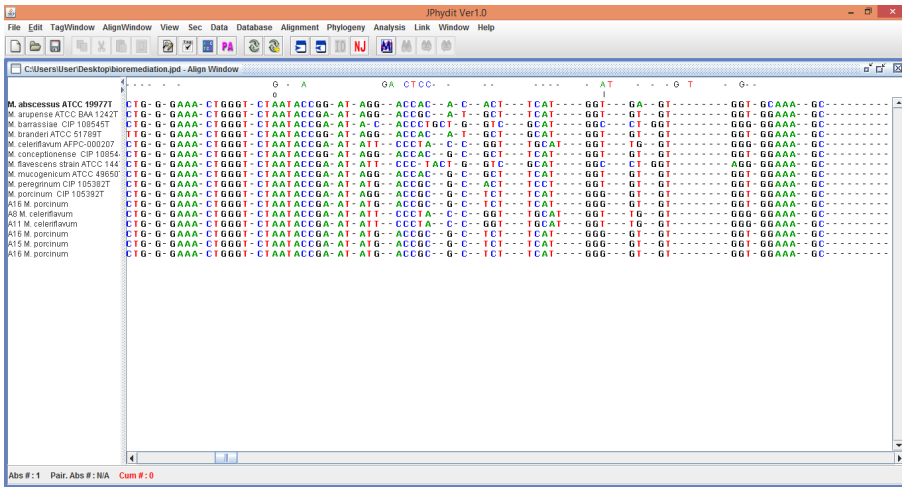

Figure 3. Alignment of the selected 16S rRNA sequencing of Iranian *Mycobacterium* isolates with of type strains of closely related mycobacteria.

The study of biodegradation activity of isolates was performed in such a way that 1 mL of the bacterial suspension equal to 0.5 McFarland (0.5 × 108 × CFU/mL) was added to a 300 mL flask containing MSM and 1% PAHs as carbon and energy sources. Then, it was incubated for 144 hours at a temperature of 30-35°C in an orbital shaker incubator (90 rpm). The samples were analyzed at 12-hour intervals to observe the growth of the bacteria and while its absorption was measuring by absorbance at 560 nm (Figure 4).

Increasing turbidity in the culture medium indicates the consumption or degradation of the substance by the strains studied.

For final confirmation of PAHs' biodegradation activity by environmental isolates of *Mycobacterium*, the standard method recommended by Bacons was used as follows (1). First, 10 ml of the MSM with PAH that had shown growth was transferred into a sterilized tube and added to 0.6 ml of tetrachlorethylene and methanol (1:100) suspension as the extraction solvent, then, the tube was vortexed for 10 seconds, and it was centrifuged at 3000 ×g for 10 minutes. The organic phase was transferred to a new tube to analyze the amount of PAH by high performance liquid chromatography (HPLC). The size of consumed PAH by isolates were analyzed by injecting 100 µL of the organic phase into the HPLC device (Manager 5000, Knauer,

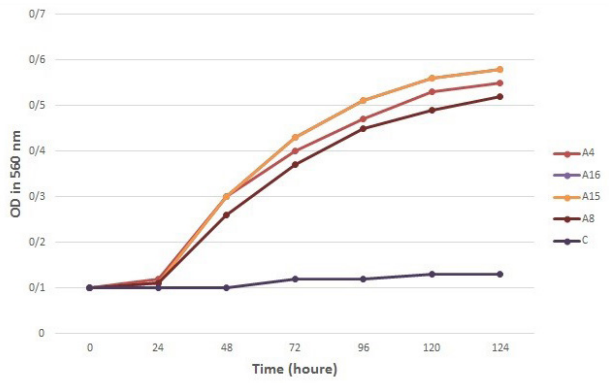

Figure 4. Growth curves of Iranian isolates of mycobacteria over 24 hours. Incubation period at 30°C in the presence of PAHs.

Germany), with C18 Ultrasep ES PAH-QC specia1 60 × 2 mm ID column, with acetonitrile and water (95:5) as mobile phase and 0.3 mL/min flow rate. The adsorption of suspension was measured at 254 nm and the PAH content of the sample was measured using the standard curve and function previously calculated using PAH standards (28).

The experiments were repeated twice and the average of the results was calculated as the final value. The following equation was used to compute the tested materials biosorption efficiency for PAHs by each strain; the results are shown in percentage (Table 1).

Results

The pH and temperature recorded for soil samples were between 5.5-8.5 and 4-30°C, respectively, and the pH and temperature of the waste water and sewage samples were recorded in the range of 6-8 and 5-18°C, respectively. Also, the amounts of total dissolved solids in water samples were recorded in the range of 400 to 1250.

From 30 environmental samples, 6 isolates (20%) were characterized as *Mycobacterium* based on the biochemical and phenotypic features, including acid-fast staining, iron uptake, pigmentation, and production of pyrazinamidase and tellurite. The presence of an hsp65 amplicon of 600 bp in size was a signature band for *Mycobacterium* genus (Table 1).

The analysis of 16SrRNA gene sequences of the environmental isolates showed that all isolates had nucleotide signatures of *Mycobacterium* at positions 70–98 (A–T), 307 (C), 293–304 (G–T), 328 (T), 631 (G), (A–T), 843 (C), 825–875 (A–T), 824–876 (T–A), and 1122–1151 (A–T) (Table 1). Based on phonotypic test and molecular data, 6 *Mycobacterium* isolates belonged to *M. porcinum* (A4, A14, A15, and A16) and *M. celeriflavum* (A8 and A12).

The relationship between *Mycobacterium* isolated in this study and the nearest mycobacterial species was supported by a high bootstrapping method in phylogenetic tree based on 16SrRNA gene designed by MEGA 8 (Figure 5).

Bioremediation analysis

The results showed that the isolates A4, A14, A15, and A16 identified as *M. porcinum* and A8 and A12 identified as *M. celeriflavum* can grow in the presence of 1 µg/ml of PAHs. For final confirmation of the use of PAHs, HPLC was utilized, the resulted curve of which showed that the strains of of *M. porcinum* and *M. celeriflavum* could degrade 70% and 90% of 1 mg/mL PAH solution in 7 days, respectively (Figure 6).

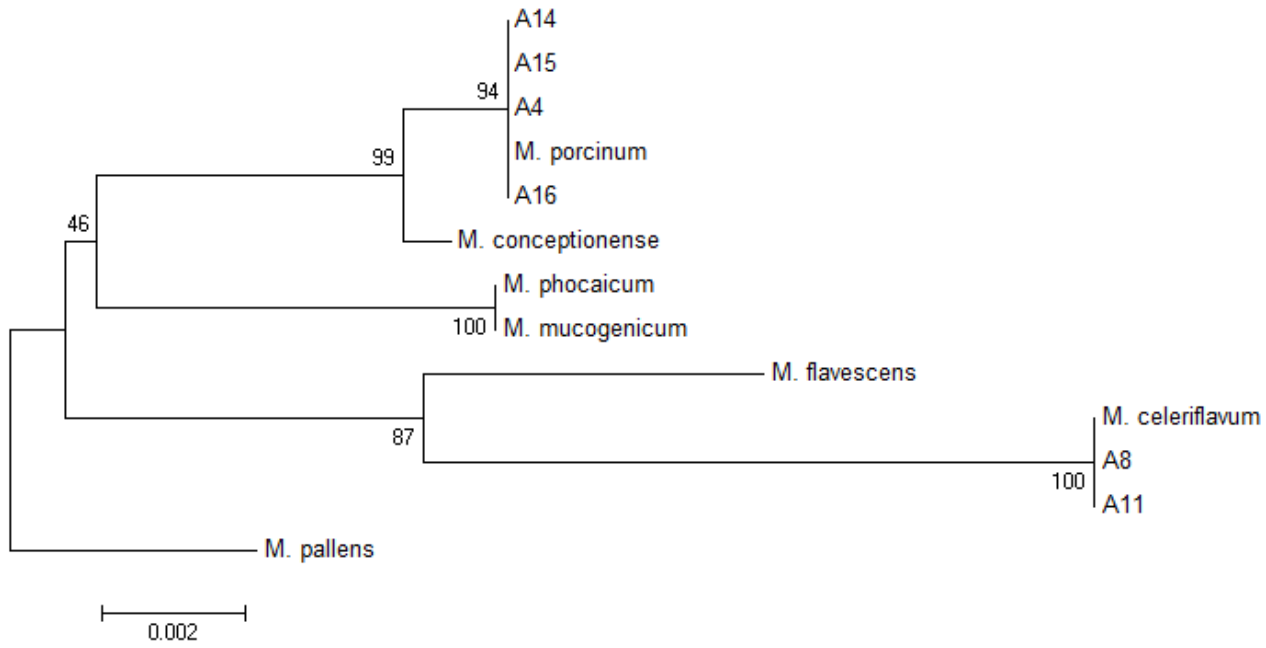

Figure 5. 16S rRNA sequence-based phylogenetic tree for Iranian biodegrading NTM isolates and the nearest validated species of mycobacteria using the neighbor-joining method. The figures at each node represent bootstrapping values. The tree was rooted with *M. pallens*.

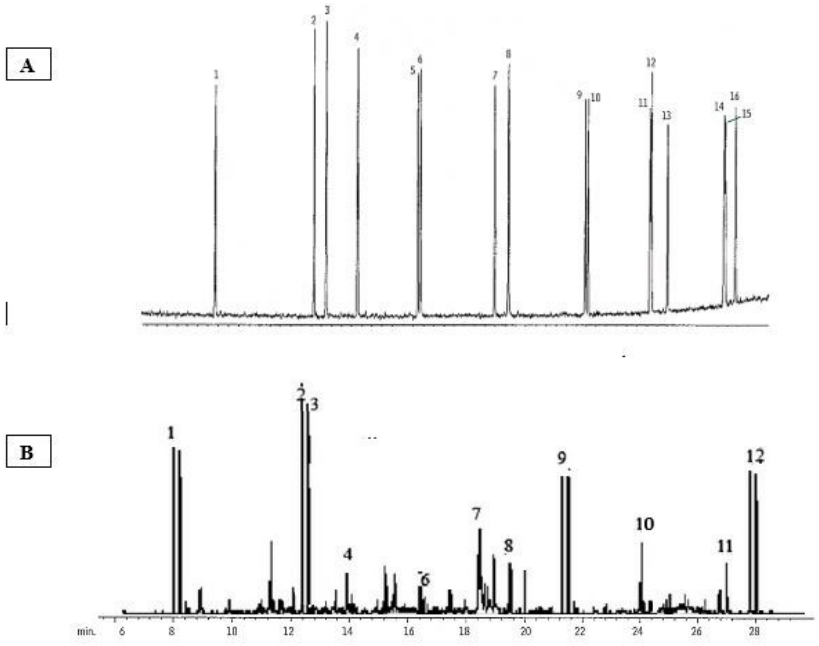

Figure 6. HPLC chromatograms of PAHs mix solution by isolate A8, (**A)** control samples, (**B)** after 144 incubation at 30°C. 1. Naphthalene, 2. Acenaphthylene, 3. Acenaphthene, 4. Fluorene, 5. Phenanthrene, 6. Anthracene, 7. Fluoranthene, 8. Pyrene, 9. Benzo[a]anthracene, 10. Chrysene, 11. Benzo[b]fluoranthene, 12. Benzo[k]fluoranthene, 13. Benzo[a]pyrene, 14. Indeno[1, 2, 3-cd]pyrene, 15. Dibenzo[a, h]anthracene

Discussion

In this study, various environmental mycobacteria (PAHs) from different ecosystems of Markazi province, which can degrade and grow in the presence of different pollutants, were screened and identified.

The present study consistent with the studies conducted in developed and developing countries on mycobacteria that have the potential for biologic purification of PAHs, indicating the presence of high amounts of highly active actinomycetes in the degradation of various contaminants in most of the environmental resources in different ecosystems and industrial centers in Markazi province.

In another study by Burghal et al in Iraq, it was reported that 86 isolates of *actinomycetes* were isolated from 150 soil samples around oil wells and refineries that can decompose petroleum compounds (29). In 2011, a study showed that a species of *Mycobacterium* isolated from soil can decompose PAHs at high concentrations (30). In a study by Shokrollahzadeh et al on oil sewage samples, 8 strains of *Mycobacterium* were isolated having the ability to biodegrade the enzyme and naphthalene (31). Berekaa successfully isolated a group of Actinomycetes including *Corynebacterium, Mycobacterium*, *Nocardia*, and *Rhodococcus*, a group of Actinomycetes, which could use rubber as carbon and energy sources, and can biodegrade these materials (32). The results of other similar studies are shown in Table 2. In this study, six isolates of *Mycobacterium* (20%) were isolated from 30 environmental samples collected from Markazi province which were belonging to 2 isolates of *M. porcinum* 4 isolates and *M. celeriflavum* 2 isolates. Then, the biodegradation ability of these two absolute species was studied according to the standard method mentioned in the procedure section and it was revealed that the strains of *M. porcinum* and Iranian *M. celeriflavum* after 7-day presence at a concentration of 1 mg PAH, can decompose 70% and 55% of PAH.

Due to this issue, purification of PAHs-contaminated areas is of great importance. Also, as most parts of the oil is hydrocarbons, their decomposition quantitatively is the most important process of oil removal from the environment. According to the results obtained, this microbial species can be used to remove this dangerous pollutant in Iran as an oil-rich region.

Conclusion

According to the results of the present study, the different ecosystem in Markazi province provides a suitable niche for actinomycetes group specially *Mycobacterium* with the capability of biodegradation of organic pollutants. Therefore, it is suggested that *Mycobacterium* species with degradation capability in these ecosystems can be used in bioremediation processes.

Acknowledgments

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Ethical issues

This study was approved by the Research Ethics Committee of Khomein University of Medical Sciences (Ethical code: IR.KHOMEIN.REC.1398.013). The authors certify that all data collected during the study are as presented in

Table 2. The results of similar studies which conducted on isolation of biodegradable *Mycobacterium*

Authors	Years	Sample	Species	Biodegradation activity	References
Guo et al	2010	Mangrove sediments	*M. austroafricanum*	PAH	(33)
Schneider et al	1996	Former coal gasification site	*Mycobacterium sp.*	Benz [a] anthracene, and benzo [a] pyrene	(34)
Kim et al	2009	Wastewater	*Mycobacterium sp.*	1, 4-dioxane	(35)
Legentil et al	2011	Hospital waste	*Mycobacterium smegmatis*	PBAT/sodium caseinate	(36)
Teniola et al	2005	Food	*Mycobacterium fluoranthenivorans*	*Fluoranthene/Aflatoxin*	(37)
Mishra et al.	2020	Oil-polluted soil	*Mycobacterium sp.*	*Phenanthrene*	(38)

this paper, and no data from the study has been or will be published elsewhere separately.

Competing of interests

The authors declare that they have no conflict of interests.

Authors' contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by SH, AA, and DA. The first draft of the manuscript was written by SH and DA, and AA and DA commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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