Original Article

Molecular Diagnosis Of Some Microorganisms From Oil-Contaminated Soil In Babylon Governorate

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Abstract

This study was designed to molecular diagnosis of different microorganisms from different soil which polluted by oil.
A new Record of Bacillus subtilis, Pseudomonas aeruginosa, Aspergillus flavus, Aspergillus niger, Aspergillus fumigatus were identified respectively, based on 16S rDNA and 18S rDNA sequencing fingerprinting data.
Also, some gene have detected like Bacillus subtilis strain RPL7 16S ribosomal RNA gene, Pseudomonas putida strain Xuyi_351_2 16S ribosomal RNA gene, Aspergillus flavus clone SF_652 small subunit ribosomal RNA gene, Aspergillus flavus clone SF_652 small subunit ribosomal RNA gene, Aspergillus niger isolate SUMS0061 18S ribosomal RNA gene, Aspergillus niger isolate SUMS0061 18S ribosomal RNA gene, Aspergillus fumigatus isolate AUMC13622 small subunit ribosomal RNA gene
The presence of bacteria and fungi in the study's polluted soils shows that microorganisms are ubiquitous, diverse, and capable of adapting to severe situations. When comparing oil-polluted soils to unpolluted soils, the characterization methods revealed a considerable decrease in microbial population, composition, and diversity.

Keywords: Molecular Diagnosis, Bacteria, Fungi, Contaminated Soil

INTRODUCTION

Petroleum (crude) oil is made up of a complex combination of hundreds of chemicals. Petroleum hydrocarbons make up 50–98% of crude oil, making them a significant component depending on the source of the oil. Petroleum hydrocarbons might be biodegraded using a variety of microbes. Bacteria, on the other hand, are key biodegradable microorganisms that play a major role in hydrocarbon decomposition. (Al-Dhabaan , 2019).
Abiotic processes such as volatilization, transformation, and adsorption to soil colloids (clay minerals and humus particles) play a key role in the apparent decontamination of mineral oil hydrocarbons in intentionally polluted soils. A portion of the mineral oil hydrocarbons that are added to soils is undetected (Lotter etal.,1992)
The uncontrolled release of carcinogenic, mutagenic, and immunotoxic petroleum chemicals into soil and groundwater presents a major hazard to human, plant, and animal health (Lin and Singh, 2008).
Liu et al. (2011) observed that the bacterial community was responsible for the degradation of the saturated and partly aromatic hydrocarbons in the early stages of remediation; however, the fungal community became prominent in the post-remediation decomposition of the polar hydrocarbons portion. Fungi, in general, are the first key players in degrading accessible pollutants and resistant polymers, thanks to a variety of extracellular enzymes and fungal hyphae.
According to Zhang et al. (2018), The Ascomycota accounted for up to 73–96% of total 18S sequences in a coking area soil; eight of the ten strains recovered from a PAHs-contaminated Pond were Ascomycetes, and they were also capable of removing anthracene.
An oilfield with a 50-year history of pollution influenced mostly the bacterial population, although fungi, mostly of this phylum, were plentiful, indicating that they were less sensitive to soil PAHs. (Zhou et al., 2017)
A microbial consortium formed of microorganisms equipped with varied metabolic capabilities and syntrophic interactions would perform better than a pure culture due to the complexity of crude oil. Several studies have shown that mixed cultures are more metabolically versatile than pure cultures when it comes to utilising hydrocarbon pollution as the only carbon source(Cerqueira et al., 2011).
22 species of bacteria, 1 algae genus, and 14 genera of fungus have been reported to use petroleum hydrocarbon as a carbon source, according to Bartha and Atlas. (Das and Chandran, 2010)

(Dilmi etal., 2017) goal was to isolate, screen, and identify hydrocarbon-degrading bacteria from oil-contaminated soil. Three samples of oil-contaminated soil were taken from the Arzew oil plant in Algeria's northwestern region. Using mineral salt medium enriched with 1% crude oil, sixteen bacterial strains were isolated, and these isolates were evaluated for their greatest degrading abilities. Using 16S rRNA gene sequence analysis, four chosen bacterial strains (P2.3, P2.2, S15.1, and E1.1) were identified based on morphological, biochemical, and molecular characteristics.
Oil-contaminated soil yielded a bacterium (named strain ZX4) capable of degrading poly aromatic hydrocarbons. The strain was identified as Sphingomonas paucimobilis based on 16S rDNA sequencing, whole cell fatty acid, and Biolog-GN analysis. The presence of GST (Glutathione S-transferase) revealed that the strain's GST had the ability to conjugate with CDNB, which might be related to PAH degradation. The existence of the GST gene was verified by the strain's PCR result. Phylogenetic study based on the gst sequence revealed that the strain was related to another strain that can breakdown PAH. (Xia Y& Min H,2003).
(Böltner etal., 2005) indicated that Hexachlorocyclohexane (HCH) is a tenacious insecticide that lingers in the environment. After enrichment from HCH-contaminated soil in Germany, three new HCH-degrading strains (DS2, DS2-2, and DS3-1) were identified. The alpha-, gamma-, and delta-isomers of HCH were efficiently destroyed by these bacteria, while strain DS3-1 also decomposed beta-HCH. Strain DS3-1 was shown to be closely linked to Sphingomonas taejonensis by 16S rDNA analysis, while strains DS2 and DS2-2 were found to be closely related to Sphingomonas flava and seven HCH-degrading germs recently isolated from HCH-contaminated Spanish soil by 16S rDNA analysis. As a result, the strains' geographic origin was not represented in their phylogenetic affiliation.
Bacteria that degrade hexachlorocyclohexane (HCH) are thought to facilitate natural attenuation of HCH pollution and have the potential to be used in active bioremediation methods. This study looked at the distribution, diversity, and substrate specificity of such bacteria in 13 soil samples from four different locations in Spain, with various degrees of HCH pollution. In 16 of the 36 enrichment cultures, hexachlorocyclohexane was removed. Communities decomposing hexachlorocyclohexane were clearly linked to HCH-contaminated soils, while populations developing on the delta-HCH isomer were exclusively detected in delta-HCH-contaminated soil. In enrichment cultures, beta-Hexachlorocyclohexane remained durable, and no indication of populations thriving on beta-HCH was seen. Nine HCH-degrading isolates were isolated from alpha- and gamma-HCH enrichment cultures, all of which were Sphingomonas spp. Isolation of organisms from delta-HCH enrichment cultures was unsuccessful (Mohn etal.,2006)

MATERIALS&METHODS

1-DNA extraction

FavorPrep Blood/ Cultured Cells Genomic DNA Extraction Mini Kit was used in this study.

2-Determination of DNA Concentration

Gel Electrophoresis was used to Analyze DNA Quality:-

3-The primers preparation

The primers were lyophilized, they dissolved in the free ddH2O to give a final concentration of 100 pmol/µl as stock solution and keep a stock at -20 to prepare 10 pmol/µl concentration as work primer suspended, 10 µl of the stock solution in 90 µl of the free ddH2O water to reach a final volume 100 µl

4-Primers used in the study 16s RNA

Table (1): The sequence of primers that used this study .

Primer	Sequence	Primer sequence	Tm (°C)	GC%	Size of Product (bp
16s RNA	**F**	**5'- AGAGTTTGATCCTGGCTCAG- 3'**	**54.3**	**50.0**	**1250bp**
	R	**5'- GGTTACCTTGTTACGACTT- 3'**	**49.4**	**42.1**	

Table (2): The Components of the Maxime PCR PreMix kit (i-Taq)

Component	Reaction size 20 µl
i-Taq TM DNA Polymerase(5U/µl)	2.5 U
dNTPs	2.5mM each
Reaction Buffer(10x)	1x
Gel Loading buffer	1x

Table (3): Reaction components of PCR.

Component	25 µL (Final volume)
Taq PCR PreMix	**5µl**
Forward primer	**10 picomols/µl (1 µl)**
Reverse primer	**10 picomols/µl (1 µl)**

DNA	**1.5µl**
Distill water	**16.5 µl**

Table (4): The optimum condition of detection

No.	Phase	Tm (°C)	Time	No. of cycle
1-	Initial Denaturation	95°C	5 min	1 cycle
2-	Denaturation -2	95°C	45sec	35 cycle
3-	Annealing	60°C	45sec	
4-	Extension-1	72°C	1min	
5-	Extension -2	72°C	5 min.	1 cycle

5-DNA extraction

FavorPrep Fungi/ Yeast Genomic DNA Extraction Mini Kit (Cat. No.: FAFYG 001)
Primers used in the study

Table (5): The sequence of primers that used this study .

Primer	Sequence	Primer sequence	Tm (°C)	GC%	Size of Product (bp)
ITS	F	**5′- TCCGTAGGTGAACCTGCGG -3′**	60.3	50 %	**600bp**
	R	**5′ TCCTCCGCTTATTGATATGC-3′**	57.8	41 %	

Table (6): The Components of the Maxime PCR PreMix kit (i-Taq)

Component	Reaction size 20 µl
i-Taq TM DNA Polymerase(5U/µl)	2.5 U
dNTPs	2.5mM each
Reaction Buffer(10x)	1x
Gel Loading buffer	1x

Table (7): Reaction components of PCR.

Component	25 µL (Final volume)
Taq PCR PreMix	**5µl**
Forward primer	**10 picomols/µl (1 µl)**
Reverse primer	**10 picomols/µl (1 µl)**
DNA	**1.5µl**
Distill water	**16.5 µl**

Table (8): The optimum condition of detection .

No.	Phase	Tm (°C)	Time	No. of cycle
1-	Initial Denaturation	95°C	5 min	1 cycle
2-	Denaturation -2	95°C	45sec	35 cycle
3-	Annealing	55°C	1 min	
4-	Extension-1	72°C	1 min	
5-	Extension -2	72°C	5 min.	1 cycle

RESULTS AND DISCUSSION

Isolation and identification of strains From contaminated soil samples, three distinct oil degrader bacteria were isolated. These strains are typically obtained using enrichment processes aiming at extracting autochthonous microbes that may use pollution as their only carbon source (Thompson et al., 2005)

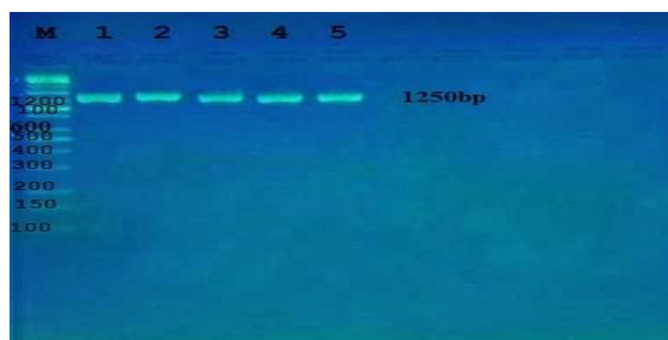

Figure (2) PCR product the band size . The product was electrophoresis on 1.5% agarose at 5 volt/cm2. 1x TBE buffer for 1:30 hours. M: DNA ladder (100).

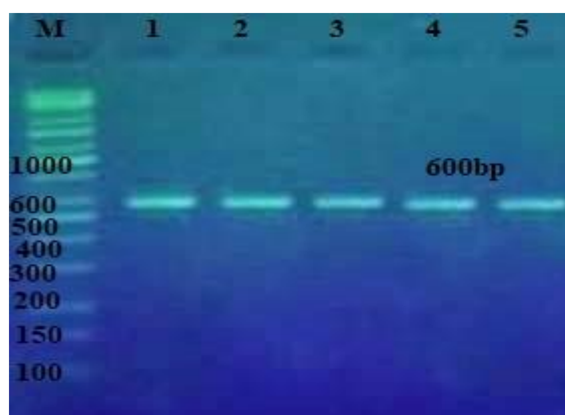

Figure (3) PCR product the band size .The product was electrophoresis on 1.5% agarose at 5 volt/cm2. 1x TBE buffer for 1:30 hours. M: DNA ladder (100).

16S rDNA sequence fingerprinting method as Bacillus subtilis, Pseudomonas aeruginosa and Bacillus cereus, These sequences were aligned with the corresponding sequences of several known hydrocarbon-degrading organisms, and the resulting phylogenetic tree indicated that these isolates were grouped into the same evolutionary trend.
These bacteria, which have evolved to contaminated environments, have enzyme systems that allow them to use hydrocarbons as their sole carbon source. In hydrocarbon-contaminated environments, different hydrocarbon-degrading microorganisms such as bacteria and archaea have been discovered. Only those strains that were highly acclimated to this severe and poisonous environment were capable of occupying this ecological niche, according to the microbial community((Whyte et al., 1997)

16S ribosomal RNA gene							
No.	Type of substitution	Location	Nucleotide	Sequence ID with compare	Sequence ID with submission	Source	Identities
1	Transvertion	190	T\A	ID: MZ191750.1	ID: ON493816.1	Bacillus subtilis	99%
2	Transvertion	384	T\G	ID: MZ191750.1	ID: ON493817.1	Bacillus subtilis	99%
	Transvertion	385	T\G				
3	Transition	324	C\T	ID: MZ191750.1	ID: ON493818.1	Bacillus subtilis	99%
	Transition	724	A\G				
	Transition	732	G\A				
4	Transvertion	678	C\A	ID: MN314434.1	ID: ON493819.1	Pseudomonas putida	99%
	Transition	701	A\G				
5	Transvertion	678	C\A	ID: MN314434.1	ID: ON493820.1	Pseudomonas putida	99%
	Transition	701	A\G				

Bacillus subtilis strain RPL7 16S ribosomal RNA gene, partial sequence
Sequence ID: MZ191750.1Length: 1398Number of Matches: 1
Range 1: 9 to 744GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
1324 bits(1467)	0.0	735/736(99%)	0/736(0%)	Plus/Plus

Query 1 GGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACC 60
Sbjct 9 68

Bacillus subtilis strain RPL7 16S ribosomal RNA gene, partial sequence
Sequence ID: MZ191750.1Length: 1398Number of Matches: 1
Range 1: 9 to 744GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
1319 bits (1462)	0.0	734/736(99%)	0/736(0%)	Plus/Plus

Query 1 GGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACC 60
Sbjct 9 68

Bacillus subtilis strain RPL7 16S ribosomal RNA gene, partial sequence
Sequence ID: MZ191750.1Length: 1398Number of Matches: 1
Range 1: 9 to 744GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
1315 bits (1457)	0.0	733/736(99%)	0/736(0%)	Plus/Plus

Query 1 GGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACC 60
Sbjct 9 68

Pseudomonas putida strain Xuyi_351_2 16S ribosomal RNA gene, partial sequence
Sequence ID: MN314434.1Length: 966Number of Matches: 1
Range 1: 65 to 710GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
1157 bits(1282)	0.0	644/646(99%)	0/646(0%)	Plus/Plus

Query 1 GGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTCCGGAAACGGG 60
Sbjct 65 124

Pseudomonas putida strain Xuyi_351_2 16S ribosomal RNA gene, partial sequence
Sequence ID: MN314434.1Length: 966Number of Matches: 1
Range 1: 65 to 710GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
1157 bits(1282)	0.0	644/646(99%)	0/646(0%)	Plus/Plus

Query 1 GGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTCCGGAAACGGG 60
Sbjct 65 124

18S rDNA sequence fingerprinting method
Generally, fungi involved in the degradation of PAHs include ligninolytic and non-ligninolytic fungi. Most of the strains isolated after the enrichment steps belonged to the phylum Ascomycota, although few Basidiomycota were isolated, Although no variations were identified among the collection depths, the total load of bacteria and fungi was lower than in

unpolluted soils: a strong and long-lasting pollution of this site may shape a microbial community adapted to the pollutants pressure, dominated by oil-using strains.

18S ribosomal RNA gene							
No.	Type of substitution	Location	Nucleotide	Sequence ID with compare	Sequence ID with submission	Source	Identities
1	Transition	85	C\T	ID: MT529928.1	ID:ON510112.1	Aspergillus flavus	99%
2	Transition	204	A\G	ID: MT529928.1	ID:ON510113.1	Aspergillus flavus	99%
	Transvertion	393	T\G				
3	Transition	371	G\A	ID: FJ011541.1	ID:ON510114.1	Aspergillus niger	99%
4	Transvertion	389	G\T	ID: FJ011541.1	ID:ON510115.1	Aspergillus niger	99%
5	Transvertion	423	A\C	ID: MT969348.1	ID:ON510116.1	Aspergillus fumigatus	99%
	Transition	439	T\C				

Aspergillus flavus clone SF_652 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence
Sequence ID: MT529928.1Length: 601Number of Matches: 1
Range 1: 47 to 588GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
974 bits(1079)	0.0	541/542(99%)	0/542(0%)	Plus/Plus

Query 1 TCATTACCGAGTGTAGGGTTCCTAGCGAGCCCAACCTCTCACCCGTGTTTACTGTACCTT 60
Sbjct 47 ....C.................... 106

Top of Form
Aspergillus flavus clone SF_652 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence
Sequence ID: MT529928.1Length: 601Number of Matches: 1
Range 1: 47 to 588GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
969 bits(1074)	0.0	540/542(99%)	0/542(0%)	Plus/Plus

Query 1 TCATTACCGAGTGTAGGGTTCCTAGCGAGCCCAACCTCCCACCCGTGTTTACTGTACCTT 60
Sbjct 47 106

Bottom of Form
Aspergillus niger isolate SUMS0061 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
Sequence ID: FJ011541.1Length: 580Number of Matches: 1
Range 1: 137 to 580GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
797 bits(883)	0.0	443/444(99%)	0/444(0%)	Plus/Plus

Query 1 TGGCCGCCGGGGGCTCTCAGCCCCGGGCCCGCGCCCGCCGGAGACACCACGAACTCTGTC 60
Sbjct 137 196

Aspergillus niger isolate SUMS0061 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
Sequence ID: FJ011541.1Length: 580Number of Matches: 1
Range 1: 137 to 580GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
797 bits(883)	0.0	443/444(99%)	0/444(0%)	Plus/Plus

Query 1 TGGCCGCCGGGGGCTCTCAGCCCCGGGCCCGCGCCCGCCGGAGACACCACGAACTCTGTC 60
Sbjct 137 196

Aspergillus fumigatus isolate AUMC13622 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence
Sequence ID: MT969348.1Length: 566Number of Matches: 1
Range 1: 66 to 552GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
870 bits(964)	0.0	485/487(99%)	0/487(0%)	Plus/Plus

Query 1 GCCTTGCTGAATTATTCACCCTTGTCTTTTGCGTACTTCTTGTTTCCTTGGTGGGTTCGC 60
Sbjct 66 125

For bacteria, changes between samples are more noticeable. The structure of the microbial community was not dependent on the collecting location; hence it was not able to form unambiguous clusters depending on soil depths. Because the total content of pollutants selects the microbial community, we can assume that a pollutants threshold has been achieved in the surface topsoil in this soil: above this level, only the selected and adapted microflora can live. Indeed, the presence of both aerobic and anaerobic bacteria decreased with the vertical soil profile in a less polluted site (Biró et al., 2014).
The results demonstrated that, contrary to the pollutant utilized, both bacterial and fungal communities mirrored those discovered after 4 weeks of enrichment, and that soil depth had no effect on the evolution of microbial communities. The isolation findings matched the HTS molecular data, demonstrating that the strains obtained resembled the enriched consortia's microbial makeup.
(Sutela etal., 2019) stated Soils sustain a wide range of species that host a wide range of viromes. We focus on viruses hosted by true fungi and oomycetes (members of the Stamenopila, Chromalveolata) inhabiting bulk soil, rhizosphere, and litter layer, and representing various ecological guilds, such as fungal saprotrophs, mycorrhizal fungi, mutualistic endophytes, and pathogens, in this minireview. Viruses that infect fungus and oomycetes have permanent intracellular nonlytic lives and spread by spores and/or hyphal connections. Almost all fungal and oomycete viruses have single-stranded or double-stranded RNA genomes, and recent research has discovered a slew of new viruses that belong to unclassified family-level groupings. Infections can be asymptomatic, useful, or harmful to the host depending on the virus-host combination. As a result, mycovirus infections may influence host dynamics by contributing to multiplex interactions.

CONCLUSIONS

used culture-independent and culture-dependent approaches to examine the evolution of bacterial and fungal communities enriched from polluted soil in this work.
The results demonstrated that, contrary to the pollutant utilized, both bacterial and fungal communities, and that soil depth had no effect on the evolution of microbial communities. The isolation results matched the molecular data, demonstrating that the strains obtained resembled the enriched consortia's microbial composition.
According to the findings, contaminated soil samples include a broad community of hydrocarbon-degrading bacteria, and these strains might be exploited for oil-contaminated soil bioremediation.

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