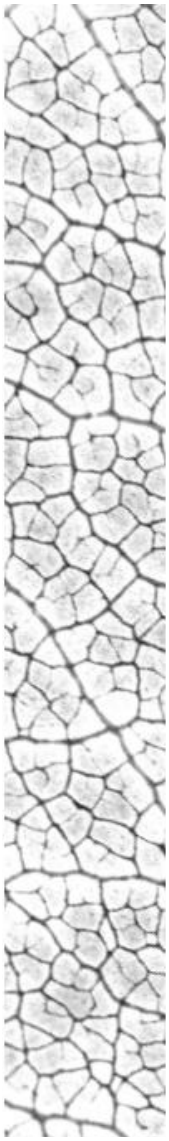

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Fungal isolates and their bioremediation for pH, chloride, tph and some toxic heavy metals

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Abstract
In this paper, an attempt was made to evaluate the effectiveness of different strains of native soil fungi (isolated from oil-contaminated environments) in the restoration of oilfield water-based drill-cuttings. Potato Dextrose Agar (PDA) was used for the isolation of fungi. About 0.5 g of fungal hyphae containing fungal isolates (after special treatment) used for polymerase chain reaction (PCR) amplification. PCR product sequencing and Basic Local Alignment Search Tool (BLAST) analysis of isolates were sent to GenBank for molecular evolutionary analyses. The evolutionary analyses and phylogenetic tree then was built by MEGA Version4. Out of 68 native hydrocarbon-degrading fungi; only four isolates were identified as the most potent strains, namely; *Aspergillus niger*-MK452260.1 (F1) *A. fumigatus*-KU321562.1 (F2); *A. flavus*-MH270609.1 (F4) and *Penicillium chrysogenum*-MK696383.1 (F3). Bio-augmentation (*in-situ*) experiments (individual/mixed cultures) were tested in 10 triplicates, excluding the control. Results (after two months of bioremediation) revealed that; F2+F4 isolates rendered the pH of drill-cuttings from strong alkaline to nearly neutrality level. F3+F4 isolates reduced chloride content by 25 folds. The isolate F2 showed the highest percentage in a reduction of total petroleum hydrocarbons (TPH). The isolate F3 showed the highest potential in the discount of lead, while the isolate F1 bioaccumulated arsenic more efficiently.

Keywords: Bioremediation, fungi, molecular characterization, drill-cuttings, pH, Cl, TPH, Pb, Hg, As

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INTRODUCTION

Maintainable advancement of petroleum assets requires suitable administration of all waste streams generated over the whole life cycle of this development beginning with the initial planning of projects and operations right through to decommissioning and site rebuilding (Baahat 2002). Iraq oil reserves, including the Kurdistan Region of Iraq (KRI), are considered as one of the largest reserves worldwide, contains about 140 billion barrels of crude petroleum (Rzgar 2017). In the oil and gasoline industry, massive portions of oily drill-cuttings and spent muds are produced annually, containing high polycyclic aromatic hydrocarbons (PAHs) and TPH content (Ramirez 2009, Qingren et al. 2011, Onwukwe and Nwakaudu 2012). These compounds are very damaging to the environment and public health due to the fact that they could be mutagenic and/or carcinogenic (Morillon 2002, Okparanma and Ayotamuno 2008, Sharif et al. 2017).

Through review from many scientific studies around the world; the major heavy metals in oil-laden soils are; Pb > Ni > V > Zn > Cd > Hg > As. Heavy metals most generally related to the poisoning of people are lead (Pb), mercury (Hg) and arsenic (As). Heavy metal poisoning may additionally take place as an end result of petroleum industrial exposure, air or water pollution, etc. (Kaewtubtim et al. 2016, Oz et al. 2019, Wang and Zhang 2017).

Bioremediation technology encompasses the controlled, practical use of micro-organisms for the breakdown of undesirable pollution (Grossi et al. 2008, Odokwuma and Dickson 2003). These various technologies rely on the biodegradation activities of micro-organisms, such as; bacteria, fungi, yeast, etc. (Spormann and Widdel 2000). The purpose of bioremediation is to degrade pollution so that the last concentrations are either undetectable or, if detectable, under the limits mounted as secure with the aid of regulatory companies (Widdel and Rabus 2001). Bioremediation of soils contaminated by way of hydrocarbons is a built-up strategy these days and has been put in exercise in various approaches such as “in-situ” or “ex-situ” applied sciences (Das and Adholeya

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2012, Das and Chandran 2001, Díaz 2008, Ite et al. 2012).

In the natural environment, a range of studies has proven that petroleum hydrocarbons are considered to be biodegraded mainly via numerous groups of bacteria and fungi (Block et al. 1991, Ite and Ibok 2019, Stroud et al. 2007). Some petroleum hydrocarbon pollution degrading microbes have the capability to biodegrade aliphatic hydrocarbons; some can biodegrade mono-aromatic or poly-aromatic hydrocarbons while a number of different hydrocarbon-degrading microbes can biodegrade resins (Sathishkumar et al. 2008, Varjani 2017). Eukaryotic organisms of fungus are among the best organisms which could degrade compounds of oil hydrocarbon. Different studies have discovered plenty of fungal species which are able for applying crude oil as the main source of energy and carbon-like *Trichoderma, Mortiecerella, Aspergillus spp., Alternaria, Talaromyces, Cephalosporium, Penicillium, Geotrichum, Fusarium* and *Cladosporium* (Ite et al. 2013), accordingly *Penicillium* and *Aspergillus* derived from oil-polluted sites would be very impressive in degradation of crude oil. However, the effectiveness of consortia/mixed cultures of different fungal strains on soil bioremediation has been much debated and is still the subject of numerous research investigations (Ite and Ibok 2019).

The overall objective of this research project (started from October 2017 and ended in August 2019) is to evaluate effectiveness of different strains of native soil bacteria and fungi, isolated from oil-contaminated environments in the bioremediation of oilfield water-based drill-cuttings, demonstrating capability of most potent strains (bacteria and fungi) (individually/mixed) in feasibility of the bioremediation efficiency on the drill cuttings pH, chlorides, nutrients concentrations, heavy metals, polyaromatic hydrocarbons (PAHs), TPH and microbial population density (heterotrophic bacteria and fungi). Finally, results supplemented by confirmatory toxicity characteristic leaching procedure (TCLP) as end-point (in this paper, only isolation, molecular identification and characterization of native soil fungal strains and their bioremediation for pH, chlorides, TPH and some toxic heavy metals, namely; Pb, Hg and As is highlighted) (full data can be obtained from the corresponding author).

MATERIALS AND METHODS

Study Area / Sample Collection

Generally, oil and gas fields in KRI (where drill-cutting samples were collected) are located in the Zagros Fold belt of Kurdistan, within the High Folds with wide synclines Zone of Iraq. The region experiences a Mediterranean semi-arid climate. The highest mean annual air temperature recorded for the years 2016, 2017 and 2018 was 47.03°C and the lowest of -4.00°C. The lands are rain-fed agricultural lands. Crops mainly found include wheat and barley, along with summer and winter vegetables.

Fig. 1. Map of oil and gas fields at KRI, location of drill-cutting sampling sites are indicated

Samples of drill-cuttings were collected from 41 drilling waste pits (GPS coordinates of the pits are not given here). The pits were distributed over five major oil and gas fields located at Erbil, Sulaimani, Duhok and Halabja provinces and Garmiyan administration at the Kurdistan region of Iraq, Iraq (**Fig. 1**).

The drill-cuttings were aseptically collected using a soil hand auger. Indicative sampling depth was around 0 to 50 cm. Each sample was made by homogenization of 3 to 4 samples taken in an area of several square meters. Duplicate samples were taken for analysis (duplicate held in reserve). The drill-cuttings were collected into sterile glass jars or/and nylon bags to preserve TPH and brought back immediately (within 24 hours of collection) for laboratory analyses, where they were homogenized again using a sterile spatula.

Enrichment of Hydrocarbon-Degrading Fungi

Soil enrichment techniques the use of Potato Dextrose Agar (PDA) has been used for the isolation of fungi. Then pure isolates were tested for the capability to grow on Bushnell Haas Media (BHS), composed of: K_2HPO_4 (1 g/l), $MgSO_4.7H_2O$ (0.2 g/l), KH_2PO_4 (1 g/l), $CaCl_2$ (0.02 g/l), $NaNO_3$ (1 g/l) and $FeCl_2$ (0.05 g/l) at 30℃ for one week (Dawoodi et al. 2015). Sixty-eight unique isolates have been selected for further research based on growth on the BHS medium. The isolated fungi were recognized using morphological and molecular strategies for this purpose. The gross morphology of the fungal increase on plates was studied with their colors. Small portions of the fungal pure culture were teased and mounted in lactophenol cotton blue stain on a clean

grease-free glass slide and covered with a clean coverslip and determined under the microscope, referencing to the Manual of Fungi Atlas (Watanabe 2002). The identities of the fungi were certified using the cultural method as well as comparing them with demonstrated representative identified by means of keys (Booth 1977, Okigbo 2009). The incidence of every fungus has calculated the usage of the formula outlined by means of Sepic et al. (1995).

Molecular Identification of Fungal Isolates

About 0.5 g of fungal hyphae was once taken from a tube containing fungal isolates. Hyphae were incubated in 100 µl lyticase solution at 30°C for 60 minutes. In order to degrade protein from the crude sample, 20 µl proteinase K was brought and incubated at 55°C for ninety min. The sample was once incubated for two hours at 65°C. Finally, about 10 µl of these samples were used for polymerase chain reaction (PCR) amplification. software used to be set in accordance with the stipulations described by means of Katoh and Standley (2013). Briefly, for the 25 µl PCR reactions, the universal primers ITS1 (5´TCC GTA GGT GAA CCT TGC GG 3´) and ITS2 (5´TCC TCC GCT TAT TGA TAT GC 3´) had been used (Ali and Abdullah 2019, Ayotamunoa et al. 2006). The M primers were; 1.5 mM $MgCl_2$, 10 µM dNTPs, and 1 x buffer. PCR was set 35 cycles and the amplification was used to be carried out at as the following condition: 94°C for 1 min; annealing at 55.5°C for 2 min and extension at 72°C for 2 min; ultimate extension at 72°C for 10 min and 4˚C incubation at the end of last cycle.

PCR Product Sequencing and BLAST Analysis

Potent strains (based on ITS1 and ITS2) were conducted using different species of *Aspergillus* and *Penicillium* commune from GenBank. The sequences were subjected to a homology search using the Basic Local Alignment Search Tool (BLAST) programmer of the National Centre for Biotechnology Information (NCBI). Based on the homology index the fungi were identified and the phylogenetic tree was constructed using NCBI. The evolutionary distances were computed using the most extreme Likelihood approach and are within the units of the number of base substitutions per site. The evolutionary analyses and phylogenetic tree were once conducted by MEGA VERSION 4 as described by Ling et al (2019).

Experimental Set-up

Out of 68 hydrocarbon-utilizing fungi (HUF); only four isolates were identified, namely; *Aspergillus niger*-MK452260 (labeled as F1 isolate); *A. fumigatus*-KU321562 (labeled as F2 isolate); *Penicillium chrysogenum*-MK696383 (labeled as F3 isolate); and *A. flavus*-MH270609 (labeled as F4 isolate) as the most potent strains. The bio-augmentation experiments (using individual/mixed fungal isolates) were carried out in 10 triplicate microcosms (plastic bowls of 35 cm by 11 cm), excluding the control (contains only soil and drill-cuttings) and then allowed to settle for about one week for the beginning of microbial activity before the addition of working solution. About 20 ml of working solution of a pure culture of F1, F2, F3 and F4 having the cell density of 7.6 x 10^{11} CFU/ml was added to each microcosm (containing 0.5 kg soil and 4 kg of drill-cuttings). Another 20 ml of working solution of mixed culture (consortium) of F1+F2, F1+F3, F1+F4, F2+F3, F2+F4, and F3+F4 isolates, having the cell density of 1.5 x 10^{12} CFU/ml was added to other microcosms (containing also 0.5 kg soil and 4 kg of drill-cuttings). It should be noted that the working solution was added to each microcosm every 14 days. While watering and mixing of a set of series of microcosms were performed as three days' interval under the temperature of 30°C over a period of 60 days. The composite samples were derived by the application of a small garden trowel at 14 days' interval for being analyzed in the laboratory.

Methods

The pH of drill-cutting samples was observed by the glass electrode pH meter (Adwa pH-Adwa Microprocessor pH meter) utilizing soil and water ratio 1:1. The chloride in drill-cuttings determined according to Haddadi and Shavandi (2013). The heavy metals were determined using Buck Model 210/211 AAS 220GF graphite Furnace and 220 AS autosampler. The method of *in-situ* was conducted according to the direct air-acetylene flame method following the condition spectrophotometer chosen for the determination of Pb, Hg, and As. Gas chromatography (EPA method number 8015) using an HP 6890 GC-MS with a flame ionization detector (FID) was used to measure the TPH concentration. Native soil samples were extracted to determine if any initial TPH existed. The hydrocarbon degradation percentage (%) and percentage (%) of occurrence of fungal isolates were measured applying the formula described by Sepic et al. (1995):

$$\text{Degradation (\%)} = a\text{-}b/a\times 100$$

Where (a) is the initial weight of underrated crude oil (control), and (b) is the weight of the remaining crude oil after degradation.

$$\text{\% of Occurrence} = a/b\times 100$$

Where (a) is the total number of each isolate, and (b) is the total number of all isolates.

Quality Assurance and Quality Control (QA/QC)

Quality assurance/control technique well-known strategies and procedures have been strictly adhered to in the course of this study. QA/QC procedures were carried out at some point of sample collection, labeling, analyses, and statistics verification. Chain of custody methods together with sample handling, transportation, logging and cross-checking in the laboratory have also been implemented.(Levchenkova et al. 2019).

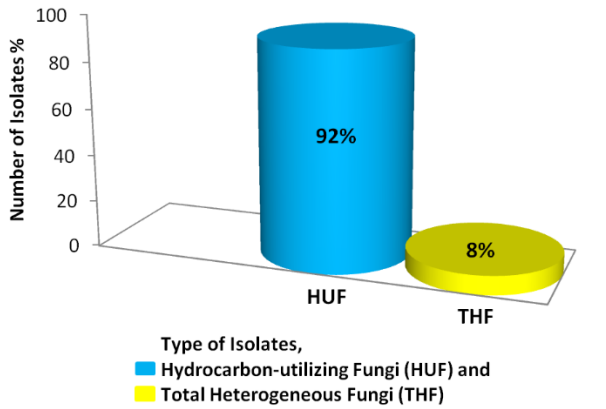


Fig. 2. Frequency of occurrence % of THF and HUF fungal isolates in oil-laden soils

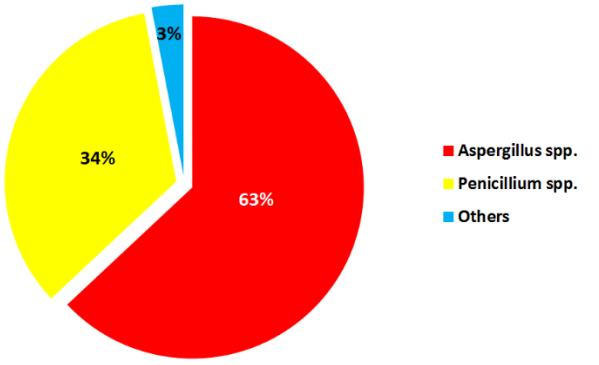


Fig. 3. Mean percentage occurrence (out of 92%) of hydrocarbon-utilizing fungal isolates

Statistical Analysis

Statistical analysis was carried out on the data generated from the fungal counts and hydrocarbon concentrations for the different treatments using ±SD and analysis of variance (ANOVA) and two way ANOVA Multiple Comparison tests, to test for the significant difference between the various treatment options at 95% (*P<0.05*) confidence.

RESULTS AND DISCUSSION

Isolation and Identification of Fungi

The hydrocarbon-utilizing fungi (HUF) isolated from oil-laden soil samples were mainly predominated by *Penicillium spp.* and *Aspergillus spp.* both occurred mostly in all the samples, while the heterogeneous isolates were mainly including; *Cladosporium spp.*, *Candida spp.*, *Mucor spp.*, *Rhizopus oryzae*, *Rhizopus spp.*, *Geotrichium spp.* and *Trichophyton spp.*, and others. The fungi isolated during this study are close to those reported previously by Walworth and Reynolds (1995). The frequency of occurrence of total heterogeneous fungi (THF) and hydrocarbon-utilizing fungi (HUF) is depicted in **Fig. 2**, it seems that; out of 68 isolates, the frequency occurrence of the HUF was higher than that of the THF in the studied oil-laden soil

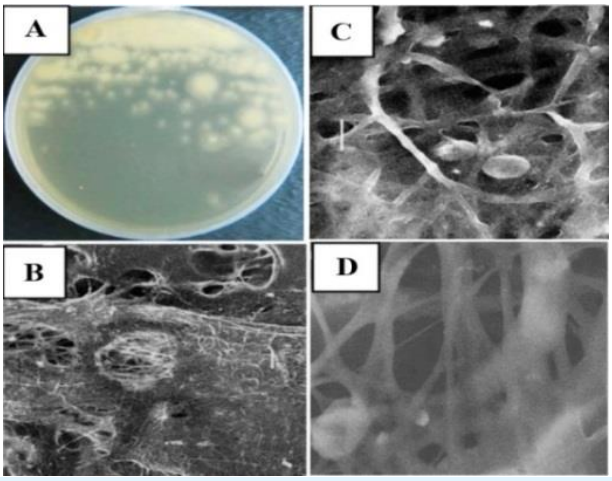


Fig. 4. Morphology of Aspergillus fumigatus strain 004, (Under scanning electronic microscope)

samples, with the frequency of occurrence of 92% for HUF and only 8% for THF. On the other hand, the mean percentage occurrence (out of the 92% of HUF) of fungal isolates was represented by; 34% for *Penicillium spp.*, 63% for *Aspergillus spp.* and 3% for other isolates (**Fig. 3**). In general, it can be concluded that HUF isolates (in contrast to other strains) are able to utilize crude oil as their sole carbon source. Similar conclusions were previously made by many authors (Bensch et al. 2012, Samuel et al. 2017).

Morphology of Isolated Fungal Strains

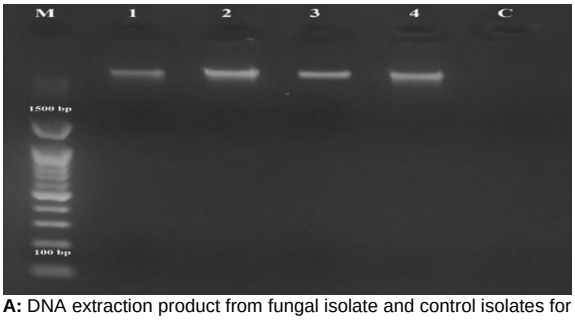
The fungal isolates were identified on the basis of morphological and molecular techniques. Genuine fungous isolates were tested under the scanning electron microscope for evaluating the morphology (**Fig. 4**). The aimed fungus was recognized by the application of characteristics of morphology and taxonomic keys presented within the success key of mycology (Watanabe 2002). **Fig. 4** shows the morphology of *Aspergillus fumigatus* strain 004 on the nutrient agar plate and different magnifications of 100X, 750X and 1000X. The colonial morphology is yellow at first but quickly becoming brown to yellow-green with radial grooves cottony and powdery colony. While, morphological characteristics are; conidia heads are large, globule and dark brown hyaline with septate hyphae.

Molecular Identification and DNA Extraction from Isolated Fungi

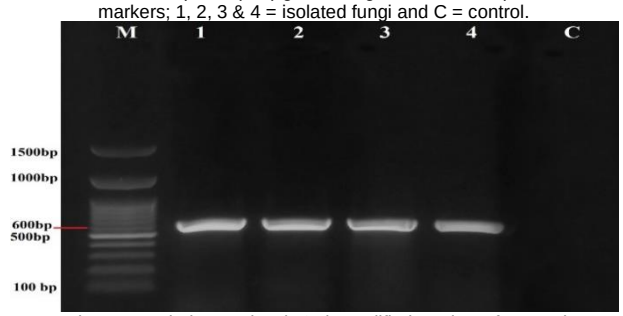
Internal Transcribed Spacer (ITS1) and (ITS2) were amplified the usage of specific primers. PCR product used to be analyzed in 2% agarose gel stained with ethidium bromide. These isolates have been molecularly identified using ITS1 and ITS2 primers as *Aspergillus* (i.e. *Aspergillus niger*, *A. fumigatus,* and *A. flavus*) and one strain belongs to *Penicillium* (i.e. *Penicillium chrysogenum*) **Fig. 5** (full partial sequencing results and pairwise alignment partial genes of the different isolated

Table 1. Identification of most potent isolated hydrocarbon-degrading fungus, along with GenBank accession number and GenBank species identification

Query Cover (%)	Identity Number (%)	GenBank Accession Number	GenBank Fungus Species Identification	Country Identification
97	98.86	MK452260.1	*Aspergillus niger* strain F3	Egypt
97	99.00	KU321562.1	*Aspergillus fumigatus* Strain 004	China
97	99.82	MK696383.1	*Penicillium chrysogenum* Strain CBS132208	China
97	98.07	MH270609.1	*Aspergillus flavus* ND103	Zimbabwe



A: DNA extraction product from fungal isolate and control isolates for Internal transcribed spacer (ITS) gene along with 600-100 bp marker. M = markers; 1, 2, 3 & 4 = isolated fungi and C = control.



B: Polymerase chain reaction (PCR) amplified products from various isolated fungi. M: markers; Lane 1: *Aspergillus niger*; Lane 2: *Aspergillus fumigatus*; Lane 3: *Penicillium chrysogenum*; Lane 3: *Aspergillus flavus* and C is the control.

Fig. 5. Agarose gel electrophoresis of PCR amplification product of fungal isolates using ITS primers

fungi can be obtained from the corresponding author). The phylogenetic tree of these 4 sequences used to be built against four distinct fungi from GenData Bank using MEGA VERSION, 4 as proven in **Fig. 6**. The most efficient tree with the sum of branch size = 36.19313893 is shown. The facts confirmed that these three species laid in the same companies with many of Aspergillus and Penicillium species from GenData Bank. Identification of the most mighty isolated hydrocarbon-degrading fungus characteristic alongside with GenBank accession quantity and GenBank fungus species identification assessed and in **Table 1**.

Total microbial DNA was once extracted from authentic soil samples and from every enrichment step of fungal culture/microcosm with the PowerLyzer Power Soil kit (MoBIO Laboratories, Inc., Carlsbad, CA, United States) following the manufacturer's instructions. DNA

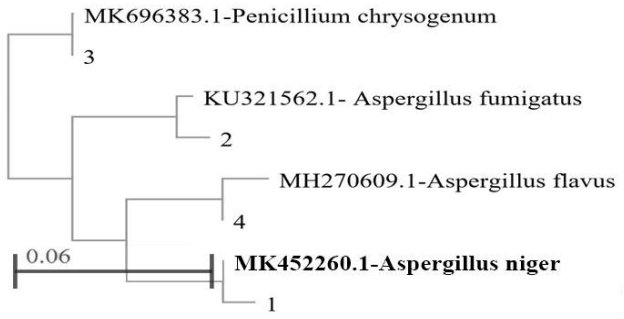


Fig. 6. An evolutionary tree for the isolated fungi based on the sequence of Internal Transcribed Spacer (ITS)

purity was once checked with electrophoresis on a 0.8% agarose gel, whilst quantification was once performed with the Quant-iTTM HS ds-DNA assay kit (Invitrogen, Paisley, United Kingdom) approach in mixture with the QuBitTM Fluorometer. Primers subsequence of *Aspergillus fumigatus* and somewhat *Penicillium chrysogenum* and two other fungi in nearly 600 bp DNA fragment of polymerase chain reaction (**Fig. 5**). The primer *Aspergillus fumigatus* has been introduced to be within the system of fungus-specific. These primers could amplify a section of the 18S rDNA sequences of *Aspergillus fumigatus* and *Penicillium chrysogenum* powerfully. **Fig. 5(A)** shows DNA extraction product from fungal isolate and control isolates for internal transcribed spacer (ITS) gene along with 600-100 bp marker, while **Fig. 5(B)** outlines polymerase chain reaction (PCR) amplified product from various fungus including; *Aspergillus niger, Aspergillus fumigatus, Penicillium chrysogenum and Aspergillus flavus.*

Dendrogram Method

Phylogenetic and alignment reconstructions were conducted by means of the BUILD Function of environment for tree exploration (ETE) V3.0.0b32 as performed on the GenomeNet (Ali and Abdullah, 2019). By the application of multiple alignments using a fast Fourier transform (MAFFT) program at the default options the process of alignment was carried out (Bensch et al. 2012). Additionally, the tree was created by the application of default parameters of Fast Tree V2.1.8. The internal transcribed spacer (ITS) region of the filamentous fungi strains was arranged and then introduced to the sequence database of GenBank with the entrance number of MK452260.1 as could be seen from the optimal evolutionary tree of the main strain given in **Fig. 6**, which shows evolutionary tree for the potent fungal isolates based on the sequence of an internal transcribed spacer (ITS), the outcome of dendrogram method for identification of isolated most potent strains of fungi is; *Aspergillus niger*-MK452260.1; *A. fumigatus*-KU321562.1; *Penicillium chrysogenum*-MK696383.1 and *A. flavus*-MH270609.1 (**Fig. 6**).

Table 2. Range of properties of the KRI untreated drill-cuttings (before bioremediation)

Property	Value*	Property	Value
pH	8.5 – 12.3	Particle size	4 µm – 15 mm
Chloride	Up to 4.0%	Gravel	1 – 4%
Hydrocarbons (TPH)	Up to 31.3%	Sand	16 – 42%
Density	1.6 – 3.2 g/cm^3	Silt	34 – 59%
Water content	18 – 36%	Clay	18 – 28%
Liquid limit	31 – 68%		

* The mean value was calculated from 150 drill-cutting samples, collected at 5 major oilfields in KRI (**Fig. 1**)

Initial Characterization of the Drill-Cuttings (Before Remediation)

Mean value (calculated from 150 drill-cutting samples, collected at 5 major oil fields in KRI, **Fig. 1**) and range of properties of the collected untreated drill-cuttings (before remediation) are shown in **Table 2**. The pH value was sharply on the alkaline side of neutrality, ranging from 8.5 to 12.3, with a common of 10.4. This shows robust alkaline wastes, which is believed to be due to chemical substances used in the course of drilling that changed the sturdy acidic nature of the cuttings to sharply alkaline. Additionally, the essential contaminants had been discovered to be barium sulfate (data is now not given here), which is a weighting agent in the drilling mud and is the main cause of the high pH of the cuttings (Colwell et al., 1977). There is no standard value recommended for the pH value of drilling wastes by Iraqi regulations (www.cwciraqpetroleum.com 2019). However, International Finance Corporation (IFC/World Bank Group, 2019) established a guideline value of 6.5–9.0 for the pH of drilling wastes, including drill-cuttings.

The concentration of chloride in untreated drill-cuttings was up to 4.0% (**Table 2**). Drill-cuttings are heterogeneous wastes that comprise mostly of hydrocarbons, heavy metals, and chlorides. One of the main types of WBD fluid additives is soluble salts such as potassium chloride (KCl). Maximum chloride awareness must be much less than 4 times the ambient awareness of fresh or brackish receiving water (Ite et al. 2013), accordingly the chloride content of drill-cuttings at the studied sites assessed as moderate.

The properties of TPH of the polluted pieces drill-cuttings are presented in **Table 2**, which comprises up to 31.3%. In accordance with the statistics of this table, it could be seen that the TPH level within the drill-cutting samples overstepped the aforementioned limit of 10.000 mg/kg fixed by the government of the Iraqi department of petroleum resources (www.cwciraqpetroleum.com). This indicates that the drill-cuttings are unsafe lands for usual applications without treatments.

The texture of drill-cuttings was clearly ranging from fine to coarse particulates (4 µm to 15 mm) and density was ranging between 1.6 to 3.2 g/cm^3. The water content of the studied drilling cuttings represented by 18 to 36%, while liquid limits ranging from 31 to 68%. Drill-cuttings are portions of rocks that are generated when holes are drilled into the earth's crust to attain the oil and gas reservoir. Depending on the type of rock formation being drilled and the drilling rig being employed for the process, these small pieces of rocks can differ in composition, size, and texture, with particle measurement ranging from sand to gravel (Das and Adholeya 20012, Ite et al. 2012).

Table 3. Mean concentration with ±SD of pH, chloride (mg/kg) and TPH (mg/kg) levels monitored during the treatment of drill-cuttings using individual/mixed cultures of bacterial isolates, after two weeks and after two months of bioremediation

Isolate + soil + drill-cuttings	After two weeks of bioremediation			After two months of bioremediation		
	pH	Chloride	TPH	pH	Chloride	TPH
F1	4.88 ± 0.31	3960 ± 261.8	11746 ± 491.0	5.78 ± 0.33	1740 ± 254.7	3235 ± 356.7
F2	5.20 ± 0.19	7850 ± 347.4	8229 ± 416.0	5.68 ± 0.42	1910 ± 295.6	1350 ± 164.0
F3	4.92 ± 0.21	9128 ± 302.7	10445 ± 437.1	6.16 ± 0.31	4481 ± 362.9	2972 ± 271.0
F4	6.00 ± 0.29	3807 ± 347.9	13127 ± 301.4	6.86 ± 0.20	2780 ± 169.3	5063 ± 241.5
F1+F2	6.18 ± 0.26	4862 ± 452.3	10856 ± 456.1	7.64 ± 0.12	1954 ± 379.4	2754 ± 197.8
F1+F3	5.94 ± 0.30	5638 ± 455.3	12814 ± 370.5	6.32 ± 0.29	1756 ± 390.8	4743 ± 388.4
F1+F4	4.72 ± 0.24	9872 ± 689.9	10922 ± 253.7	6.40 ± 0.26	1540 ± 156.7	2749 ± 155.5
F2+F3	5.90 ± 0.32	7022 ± 265.8	8808 ± 558.1	7.54 ± 0.33	1280 ± 121.6	1581 ± 191.0
F2+F4	4.70 ± 0.17	4385 ± 205.9	11293 ± 168.8	7.70 ± 0.18	1200 ± 464.7	3641 ± 128.0
F3+F4	6.10 ± 0.31	12763 ± 277.0	12610 ± 539.4	7.54 ± 0.19	1008 ± 214.9	3829 ± 227.0
Mean (Control):	pH=12.86±0.02, Cl=24850±154.1 and TPH=16393±115.8					

Bioremediation

In this study, four strains were confirmed for biodegradation ability of oil-contaminated drill- cuttings, namely; *Aspergillus niger*-MK452260.1; *A. fumigatus*-KU321562.1; *Penicillium chrysogenum*-MK696383.1 and *A. flavus*-MH270609.1. In general, the degree of degradation of hydrogen ion concentration (pH), Chlorides (Cl) and total petroleum hydrocarbons (TPH) over 2 weeks and 2 months of bioremediation showed distinct variations by the four potent fungal isolates (individual/mixed cultures) as depicted in **Table 3**. Statistical analysis revealed no significant differences ($P<0.05$) in the degradation of the oil-contaminated drill-cuttings by the isolates of concern.

Hydronium Ion Concentration (pH)

From **Table 3**, it seems that the consortium of F2+F4 isolates (mixed culture of *Aspergillus fumigatus*-KU321562.1 and *A. flavus*-MH270609.1) rendered the pH of drill-cuttings from strong alkaline from the mean of 12.86±0.02 (at the control samples) into acidic mean of 4.70±0.17 within the first 2 weeks of bioremediation, meanwhile, the pH value gradually increased to nearly neutrality level from the mean of 7.70±0.18 after 2 months of bioremediation. Results of TPH removal rate (%) and fungal growth rate as given in **Table 5** showed

Table 4. Total petroleum hydrocarbon (TPH) removal rate (%) and fungal growth rate after two weeks and two months of bioremediation

Fungal isolate	Percentage of total petroleum hydrocarbon removal rate after two weeks of bioremediation	Fungal growth rate × 10^4 after two weeks	Percentage of total petroleum hydrocarbon removal rate after two months of bioremediation	Fungal growth rate× 10^5 after two months
F1	28.34	0.3	80.26	4.0
F2	49.80	0.6	91.76	7.9
F3	36.28	0.4	81.87	5.7
F4	19.92	0.1	69.11	4.9
F1+F2	33.77	0.1	83.20	2.0
F1+F3	21.83	0.7	71.06	4.5
F1+F4	33.37	1.8	83.23	3.6
F2+F3	46.26	1.0	90.35	4.2
F2+F4	31.11	1.2	77.78	4.8
F3+F4	23.07	1.1	76.64	5.7

Fungal growth control before bioremediation 1.2 ×10^3

that the samples with initial pH of 6.0 had the greatest TPH removal rate (%) furthermore had the highest percentage increase in fungal growth rate after two months of bioremediation (**Table 3** and **Table 4**).

pH is very integral in regulating the bodily and chemical prerequisites of soil and influences the mineral constituents of soil available to the functioning of soil organisms (including fungi). The pH of soil reduced after inoculation with fungal isolates (individual/mixed cultures), within the first two weeks, but the values were slightly increased after two months of bioremediation. It has been reported that the decrease in pH values of drill-cuttings, during the early stage of the bioremediation by fungal isolates could be from high metabolic activities which possibly resulted in the production of acidic intermediate metabolites in the system leading to decreases in the pH (Walworth and Reynolds, 1995), moreover aerobic and anaerobic biodegradation of aliphatic and aromatic hydrocarbons leads to formation of organic acids (Ite and Ibok 2019, Varjani 2017). While the gradual increase afterword (i.e. after two months of bioremediation) can be attributed to the subsequent release of intermediate final products that probably had rising effects on the pH of the treatment sets (Ite et al. 2013).

Chloride Content

The isolate of F4 alone (pure culture of *A. flavus*-MH270609.1) reduced the chloride content from the mean of 24850±154 mg/kg (at the control samples) to only mean of 3807±347 mg/kg within the first two weeks of bioremediation. While, the consortium of F3+F4 isolates (mixed culture of *Penicillium chrysogenum*-MK696383.1 and *A. flavus*-MH270609.1) showed the highest chloride biodegradation activity, and showed that they are capable of degrading and/or utilizing chloride content from the mean of 24850±154 mg/kg (at the control samples) to the mean of 1008±214.9 mg/kg after two months of bioremediation (**Table 3**). The observance of high fungal counts in tests containing consortiums indicated that a high amount of

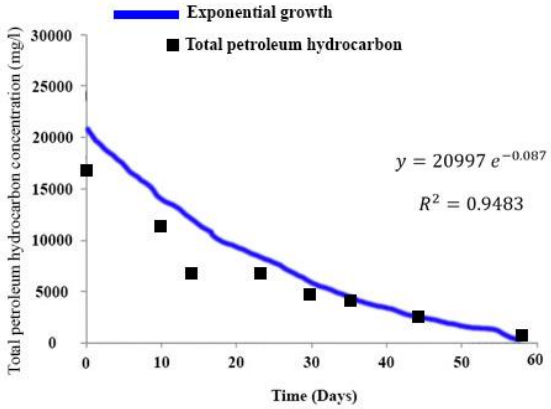


Fig. 7. TPH growth model using controlling pollution of oily hydrocarbon

biodegradation of chloride content can be achieved by employing culture containing consortium of isolates rather than single isolates. Maximum chloride content ought to be much less than 4 instances the ambient attention of clean or brackish receiving water (IFC/World Bank Group 2019).

The negative impact of high concentrations of chloride (such as $CaCl_2$, $MgCl_2$, KCl, etc.) on the growth of some fungal isolates (among them; *Penicillium roqueforti* and *Aspergillus niger*) is of well-known (Haddadi and Shavandi 2013). On the other hand, some fungal species are more tolerant of higher chloride concentrations. However, different studies indicated that there are intraspecific variations in chloride tolerance of some other fungal species (Al-Mailem et al. 2013). The same can be concluded for *Penicillium chrysogenum*-MK696383.1 and *A. flavus*-MH270609.1 for this work.

Total Petroleum Hydrocarbons (TPH)

Among evaluated fungal strains, the isolate F2 alone (*Aspergillus fumigatus*-KU321562.1) showed highest potential in reduction of TPH from the mean of 16393±115.8 mg/kg (at the control samples) to the mean of 8229±416.0 mg/kg within the first two weeks and further reduced TPH to the mean of 1350±164.0 mg/kg after two months of bioremediation (**Table 3**). This showed that the isolate F2 alone can degrade the TPH relatively better than the remainder consortia of concern (mixed cultures). However, as shown in **Table 4**, the isolate F2 alone was able to remove 91.76 % of TPH at the evaluated fungal growth rate of 7.9 × 10^5 after two months of bioremediation.

On the other hand, the consortium of F2+F3 isolates (mixed cultures of *Aspergillus fumigatus*-KU321562.1 and *Penicillium chrysogenum*-MK696383.1) showed highest potential in reduction of TPH from the mean of 16393±115.8 to the mean of 8808±558.1 mg/kg (compared with other consortia) within the first two weeks and reaching the mean of 1581±191.0 mg/kg after two months of bioremediation. TPH growth model using controlling pollution of oily hydrocarbon is given in **Fig. 7**, which shows that the exponential growth of fungal

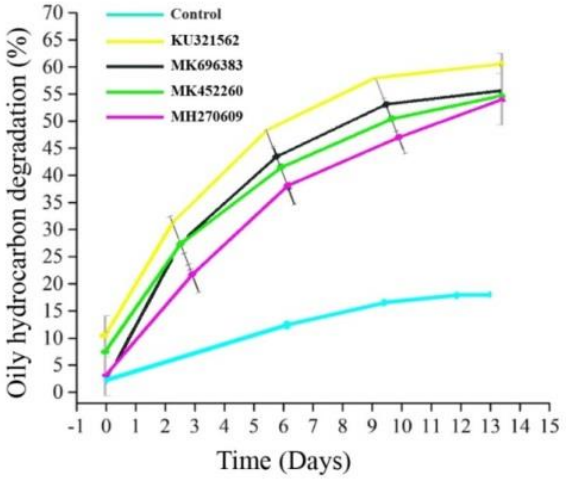


Fig. 8. Oily hydrocarbon degradation (%) via applied fungal strains

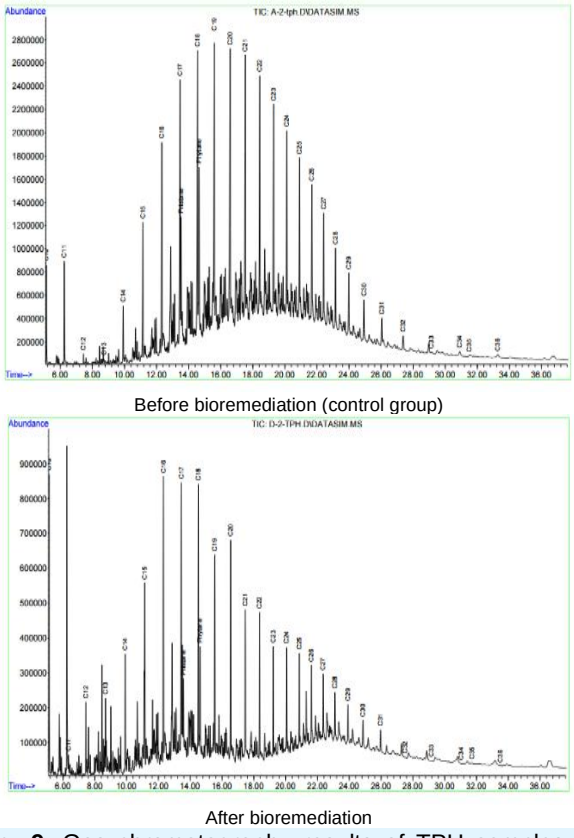


Fig. 9. Gas chromatography results of TPH samples by Aspergillus fumigatus-KU321562

isolates decreases to zero in the day 58. Oily hydrocarbon degradation *via* applied fungal strains is depicted in **Fig. 8**, from the figure it is obvious that the most hydrocarbon-degrading/utilizing fungal isolates are; *Aspergillus fumigatus*-KU321562.1> *Penicillium chrysogenum*-MK696383.1> *A. niger*-MK452260.1> *A. flavus*-MH270609.1, respectively in their order of TPH degradation during the current study.

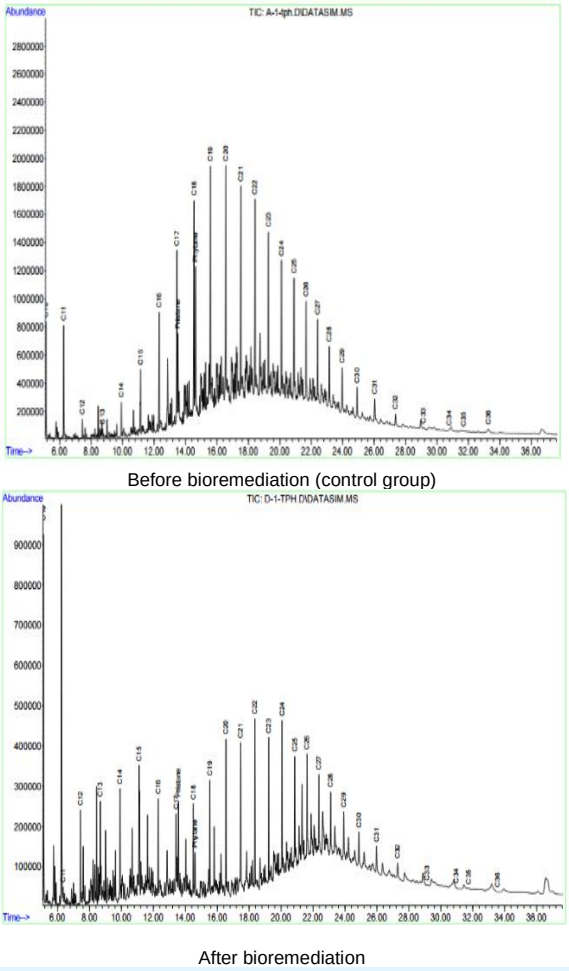


Fig. 10. Gas chromatography results of TPH samples by the consortium Aspergillus fumigatus-KU321562 and Penicillium chrysogenum-MK696383 isolates

The interpretation of hydrocarbon chromatographic results for bioremediation of drill-cuttings was examined at a sample detection limit of 0.001 to 0.01 mg per sample. Quantification and interpretation of TPH in bio-remediated drill-cutting samples by a GC/FID method (only results of two most potent fungal isolates) are illustrated in **Fig. 9**, namely; *Aspergillus fumigatus*-KU321562.1 (F2) isolate (alone) and **Fig. 10** for the consortium of *A. fumigatus*-KU321562.1 *and Penicillium chrysogenum*-MK696383.1 (F2+F3) isolates, before (at control sample) and after treatment of biodegradation.

The chemical composition of petroleum products is complicated and might also alternate over time following release into the environment. The GC-based methods normally cannot quantitatively detect compounds below C6 due to the fact these compounds are particularly unstable and interference can occur from the solvent height (Srivastava et al. 2014). Results showed the separation of a wide range of carbon numbers in the TPH from C_{11} to C_{36} (measurable TPH range). The carbon fractions detected in oil-contaminated drill-cuttings were mainly; gasoline from C_{11} to C_{12}, kerosene

C_{11} to C_{17}, diesel C_{11} to C_{24}, fuel oils C_{12} to $C_{25\text{-}30}$, lube oils and heavier products C_{20} to C_{36}. In the herbal environment, a number of research have proven that petroleum hydrocarbons are regarded to be biodegraded frequently with the aid of a various crew of bacteria, fungi, and yeast, proving that indigenous microbial communities in the hydrocarbon-impacted drill cuttings have the natural capacity to degrade TPHs when you consider that they ought to use crude oil factors as a supply of carbon and energy (Chandra et al. 2013). Biodegradation is the generic term used to describe the biological conversion, disintegration or transformation of natural contaminants by way of fungi, bacteria or different organic organisms to products that are normally lower in free energy (Heider et al. 1998; Spormann and Widdel 2000). Biodegradation involves either partial or whole mineralization of environmental natural contaminants by complex, genetically regulated physiological reactions catalyzed largely by using microorganisms (Widdel and Rabus 2001).

Moreover, petroleum hydrocarbons range in their susceptibility to microbial assaults based on their molecular structures and frequent differences in their susceptibility (Das and Chandran 2011, Ite and Semple 2012). In practice, microbial degradation of petroleum hydrocarbon–chemical wastes and/or organic contaminants in soils are strictly limited by a variety of elements (Spormann and Widdel 2000, Widdel and Rabus 2001). The range and extents of biodegradation and microbial increase in soil are influenced by way of a range of abiotic factors, together with the complexity and attention of the organic contaminant mixtures, contaminants bioavailability and/or bio-accessibility, and natural contaminants interactions in soil, natural matter, temperature, pH, availability of vitamins (particularly nitrogen and phosphorus) (data is not given here), soil moisture level, availability of oxygen, concentration of organic contaminant of concern and redox potential. Similar conclusions have been made by using many authors, however, they have simply discussed some of the most important elements that often affect the microbial degradation of petroleum hydrocarbons in the soil environment (Das and Chandran 2011, Ite and Semple 2012, Srivastava et al. 2014).

Heavy Metals

Lead (Pb)

From **Table 5**, the isolate F3 alone (*Penicillium chrysogenum*-MK696383.1) decreased the lead (Pb) content from the mean of 26.70±4.8 mg/kg (at the control samples) to the mean of 10.99 ±3.3 mg/kg within the first two weeks of bioremediation, while the same isolate further decreased the lead content to the mean of 3.96±1.0 mg/kg after the two months of bioremediation. On the other hand, the consortium of F1+F3 isolates (the mixed culture of *Aspergillus niger*-MK452260.1 and *Penicillium chrysogenum*-MK696383.1) showed highest potential reduction in lead to the mean of 11.71±2.9 mg/kg (compared with initial concentration in control) after two weeks of bioremediation, while the consortium of F2+F4 (mixed cultures of *A. fumigatus*-KU321562.1 and *A. flavus*-MH270609.1) showed the highest capability in removal of lead from the mean of 26.70±4.8 to the mean of 6.20±1.8 mg/kg after two months of bioremediation. This is not unconnected with the possibility that *Penicillium chrysogenum*-MK696383.1 may have different responses, adaptive mechanism and bioremediation approaches to lead in polluted soil (Oz et al. 2019). Furthermore, the rate of lead in the drilling waste samples relay on several factors including pH, temperature, formation solids, type of based muds, and mineral oils (Oz et al. 2019, Wang and Zhang 2017).

Table 5. Mean concentration (mg/kg) with ±SD of Lead (Pb), Mercury (Hg) and Arsenic (As) levels monitored during the treatment of drills cuttings using individual/mixed cultures of bacterial isolates, after two weeks and after two months of bioremediation

Isolate + soil + drill-cuttings	After two weeks of bioremediation			After two months of bioremediation		
	pb	Hg	As	pb	Hg	As
F1	12.93 ± 3.3	N.D	3.82 ± 1.2	4.90 ± 1.0	N.D	0.98 ± 1.4
F2	17.00 ± 2.1	N.D	4.49 ± 0.6	8.09 ± 2.0	N.D	2.42 ± 0.7
F3	10.99 ± 3.3	N.D	4.18 ± 0.6	3.96 ± 1.0	N.D	2.06 ± 0.4
F4	15.07 ± 3.4	N.D	4.95 ± 1.4	7.85 ± 2.6	N.D	1.85 ± 0.8
F1+F2	13.29 ± 4.4	N.D	5.26 ± 0.7	6.77 ± 1.4	N.D	2.67 ± 0.4
F1+F3	11.71 ± 2.9	N.D	6.60 ± 1.2	9.46 ± 1.9	N.D	2.49 ± 0.4
F1+F4	15.27 ± 4.2	N.D	5.09 ± 0.8	8.82 ± 2.3	N.D	2.12 ± 0.5
F2+F3	15.41 ± 3.6	N.D	6.73 ± 0.6	8.19 ± 2.2	N.D	3.24 ± 1.0
F2+F4	14.35 ± 4.9	N.D	5.61 ± 0.7	6.20 ± 1.8	N.D	4.07 ± 0.5
F3+F4	13.82 ± 3.6	N.D	7.30 ± 0.6	7.86 ± 2.5	N.D	4.75 ± 0.8
Mean (Control):	pb = 26.70±4.8, Hg = 00.00 and As =11.67±1.7					
	N.D means undetectable at 0.001 mg/kg detection limit					

Mercury (Hg)

Mercury content was undetectable (below the detection limit of 0.001 mg/kg) before and after bioremediation treatment (**Table 5**). Occurrences of some heavy metals (such as mercury, arsenic, etc.) in drilling wastes are noticeably associated with sources of oil-based muds cuttings (Wang and Zhang 2017), furthermore, mercury in the drilling waste samples ordinarily originate from the lube oil and grease. Similar preceding research detected zero mercury content in WBM drill-cuttings in distinctive components of the world (Oz et al. 2019).

Arsenic (As)

Concerning arsenic (As), **Table 5** showed that; the isolate F1 alone (*Aspergillus niger*-MK452260.1) degraded arsenic content from the mean of 11.67±1.7 mg/kg (at the control samples) to the mean of 3.82±1.2 mg/kg within the first two weeks of bioremediation,

meanwhile the same isolate alone further decreased the arsenic content from the mean of 0.98±1.4 mg/kg after two months of bioremediation. On the other hand, the consortium of F1+F4 isolates (the mixed culture of *Aspergillus niger*-MK452260.1 and *A. flavus*-MH270609.1) showed highest potential reduction in arsenic to the mean of 5.09±0.8 mg/kg (compared with initial concentration) after two weeks of bioremediation, while the same consortium further decreased arsenic content to the mean of 2.12±0.5 mg/kg after two months of bioremediation. There is the probability that the fungi isolate first bio-accumulates and immobilized heavy metals (including As) to some extent in their cells before biodegradation set in. The breakdown of heavy metals in the fungal cells of isolates may be brought on when the degrees of the heavy metals threaten the fungal survival specifically in confines or constrained environment. To what extent the fungal traces can bio-accumulates heavy metals and the triggering mechanisms for the breakdown of heavy metals are a remember for in addition studies (Kaewtubtim et al. 2016, Oz et al. 2019, Wang and Zhang 2017).

CONCLUSION

From onset results, the following conclusions can be made:

- Out of 68 native hydrocarbon-degrading/utilizing soil fungi; only four isolates were identified (based on morphology and gene sequence techniques) as the most potent strains, namely; *Aspergillus niger*-MK452260.1; *A. fumigatus*-KU321562.1; *A. flavus*-MH270609.1 and *Penicillium chrysogenum*-MK696383.1.
- The mixed cultures of *Aspergillus fumigatus*-KU321562.1 and *A. flavus*-MH270609.1 capable to render the pH of drill-cuttings from strong alkaline to nearly neutrality level after 2 months of bioremediation.
- The mixed cultures of *Penicillium chrysogenum*-MK696383.1 and *A. flavus*-MH270609.1 showed the highest potential in chloride biodegradation, they are able to degrade and/or utilizing chloride content in drill-cuttings by 25 folds within the two months of bioremediation.
- The isolate *Aspergillus fumigatus*-KU321562.1 showed the highest potential in reduction of TPH after two months of bioremediation and removed 91.76 % of TPH at the evaluated fungal growth rate of 7.9 × 105 after two months of bioremediation; furthermore, it can degrade TPH relatively better than the mixed cultures with other consortia of concern.
- The isolate *Penicillium chrysogenum*-MK696383.1 selected as the best strain for lead bioremediation/reduction in oil-laden soils (i.e. drill-cuttings). However, the isolate alone decreased the lead (Pb) content to 3.96 mg/kg in drill-cuttings, compared to the initial concentration of 26.70 mg/kg (at the control samples) after the two months of bioremediation.
- Concerning arsenic (As), the isolate *Aspergillus niger*-MK452260.1 reduced the arsenic content from 11.67 mg/kg (at the control samples) to 0.98 mg/kg after two months of bioremediation.

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REFERENCES

Ali MK, Abdullah SMA (2019) Morphological and molecular identification of *Capoeta trutta* (cyprinidae) and *Planiliza abu* (mugilidae) freshwater fish in Sulaimani governorate, Iraq. – Applied Ecology and Environmental Research 17(4): 7439-7451.

Al-Mailem D, Eliyas M, Radwan S (2013) Oil-bioremediation potential of two hydrocarbon clastic, diazotrophic marinobacter strains from hypersaline areas along the Arabian Gulf coasts. – Extremophiles 17: 463-481.

Ayotamunoa MJ, Kogbaraa RB, Ogajib SOT (2006) Bioremediation of a crude-oil polluted agricultural-soil at Port Harcourt, Nigeria. – Applied Energy 83(11): 1249-1257.

Baahat H (2002) Managing waste in exploration and production activities of the petroleum industry. – Environmental Advisor, SENV 1: 1–37.

Bensch K, Braun U, Groenewald JZ (2012) The genus *Cladosporium*. – Journal of Mycology 72(1): 391-401.

Block R, Allworth N, Bishop M (1991) Assessment of Diesel Contamination in Soil. – In Calabrese, E., Kostecki, P. (ed.) Hydrocarbon Contaminated Soils. MI: Lewis Publishers, Chelsea.

Booth CH. (1997) Laboratory Guide to the Identification of Major Species. – Kew, UK: Commonwealth Mycological Institute.

Chandra S, Sharma R, Singh K, Sharma A (2013) Application of bioremediation technology in the environment contaminated with petroleum hydrocarbon. – Annals of Microbiology 63(2): 417-431.

Colwell RR, Walker JD (1997) Ecological aspects of microbial degradation of petroleum in the marine environment. – CRC Critical Review in Microbiology 5(4): 423-445.

Das M, Adholeya A (2012) Role of Microorganisms in Remediation of Contaminated Soil. – In Satyanarayana, T., Johri, B. N. (ed.) Microorganisms in Environmental Management. Dordrecht: Springer Netherlands.

Das N, Chandran P (2011) Microbial degradation of petroleum hydrocarbon contaminants: an overview. – Biotechnology Research International 1: 810-941.

Dawoodi V, Madani M, Tahmourespour A (2015) The study of heterotrophic and crude oil-utilizing soil fungi in crude oil contaminated regions. – Journal of Bioremediation and Biodegradation 6(2): 12-26.

Díaz E (2008) Microbial Biodegradation: Genomics and Molecular Biology: Caister Academic Press.

Grossi VC, Cravo-Laureau R, Guyoneaud A, Ranchou-Peyruse A, Hirschler-Réa B (2008) Metabolism of n-alkanes and n-alkenes by anaerobic bacteria: A summary. – Organic Geochemistry 39(8): 1197-1203.

Haddadi A, Shavandi M (2013) Biodegradation of phenol in hypersaline conditions by *Halomonas sp*. strain PH2-2 isolated from saline soil. – International Bio-deterioration and Bio-degradation 85: 29-48.

Heider J, Spormann AM, Beller HR, Widdel F (1998) Anaerobic bacterial metabolism of hydrocarbons. – FEMS Microbiology Reviews 22(5): 459-473.

Ite AE, Ibok UJ (2019) Role of plants and microbes in bioremediation of petroleum hydrocarbons contaminated soils. – International Journal of Environmental Bioremediation and Biodegradation 7(1): 1-19.

Ite AE, Ibok UJ, Ite MU, Petters SW (2013) Petroleum Exploration and Production: Past and Present Environmental Issues in Nigeria's Niger Delta. – American Journal of Environmental Protection 1(4): 78-90.

Ite AE, Ibok UJ, Ite MU, Petters SW (2013) Petroleum exploration and production: past and present environmental issues in Nigeria's Niger Delta. – American Journal of Environmental Protection 1(4): 78-90.

Ite AE, Semple KT (2012) Biodegradation of Petroleum Hydrocarbons in Contaminated Soils. – In Arora, R. (ed.) Microbial Biotechnology. Wallingford, Oxfords hire CAB International.

Kaewtubtim P, Meeinkuirt W, Seepom S, Pichtel J (2016) Heavy metal phytoremediation potential of plant species in a mangrove ecosystem in Pattani bay, Thailand. – Applied Ecology and Environmental Research 14(1): 367-382.

Katoh K, Standley DM (2013) MAFFT multiple sequence alignment software VERSION 7: improvements in performance and usability. – Molecular Biology Evolution 30(4): 772-780.

Levchenkova, T. V., Platonova, R. I., Tarasov, A. E., Verma, P., Ammosova, L. I., Nikitina, A. E., & Olesov, N. P. (2019). Socio-Pedagogical Significance of the" gto" Complex in the Formation of Healthy Population. Journal of Environmental Treatment Techniques, 7(4), 572-575.

Ling Z, Zhen YD, Mei M (2019) Cleaning up of contaminated soils by using microbial remediation: A review and challenges to the weaknesses. – Journal of Biomedical Science and Research 2(3): 318-327.

Morillon AJ, Vidalie US, Hamzah SS, Hadinoto EK (2002) Drilling and Waste Management, SPE 73931. – Presented at the SPE International Conference on Health, Safety, and the Environment in Oil and Gas Exploration and Production.

Odokwuma LO, Dickson AA (2003) Bioremediation of crude oil polluted tropical mangrove environment. – Journal of Applied Science and Environmental Management 7(2): 23-29.

Okigbo RN (2009) Mycoflora and production of wine from the fruit of soursops. – International Journal of Wine Research 1:1-9.

Okparanma R, Ayotamuno M (2008) Predicting chromium (VI) adsorption rate in the treatment of liquid-phase oil-based drill-cuttings. – Journal of Environmental Science and Technology 2(4): 068-074.

Onwukwe SI, Nwakaudu MS (2012) Drilling wastes generation and management approach. – International Journal of Environmental Science and Development 3(3):184-191.

Oz N, Kadizade G, Yurtsever M (2019) Investigation of heavy metal adsorption on micro-plastics. – Applied Ecology and Environmental Research 17(4): 7301- 7310.

Qingren W, Shouan Z, Yuncong L (2011) Potential approaches to improving biodegradation of hydrocarbons for bioremediation of crude oil pollution. – Journal of Environmental Protection 2: 47-55.

Ramirez PJ (2009) Reserve Pit Management: Risks to Migratory Birds. US Fish and Wildlife Service Region 6, Environmental Contaminant Program, Cheyenne, Wyoming, USA.11.

Rzger AA (2017) Heavy Oil in Iraq: Review. –Journal of Scientific and Engineering Research 4(8): 134–141.

Samuel V, Enrico E, Stefano G (2017) Fine-scale spatial distribution of orchid mycorrhizal fungi in the soil of host-rich grasslands. – New Phytologist 213: 1428-1439.

Sathishkumar M, Binupriya AR, Baik SH (2008):Biodegradation of crude oil by individual bacterial strains and a mixed bacterial consortium isolated from hydrocarbon-contaminated areas. – Clean 36(1): 92-96.

Sepic E, Leskovsek H, Trier C (1995) Aerobic bacterial degradation of selected poly-aromatic compounds and n-alkanes found in petroleum. – Journal of Chromatography A 697(1-2): 515-523.

Sharif MD, Nagalakshimi NVR, Srigowri RS, Vasanth G, Sankar K (2017) Drilling waste management and control the effects. – Journal of Advanced Chemical Engineers 7: 1–9.

Spormann AM, Widdel F (2000) Metabolism of alkylbenzenes, alkanes, and other hydrocarbons in anaerobic bacteria. – Biodegradation 11(3): 85-105.

Srivastava J, Naraian R, SKalra JS, Chandra H (2014) Advances in microbial bioremediation and the factors influencing the process. – International Journal of Environmental Science and Technology 11(6): 1787-1800.

Stroud JL, Paton GI, Semple KT (2007) Microbe-aliphatic hydrocarbon interactions in soil: implications for biodegradation and bioremediation. – Journal of Applied Microbiology 102(5): 1239-1253.

Varjani SJ (2017) Microbial degradation of petroleum hydrocarbons. – Bioresource Technology 223: 277-286.

Vidali M (2001) Bioremediation, an overview. – Pure and Applied Chemistry 73(7): 1163-1172.

Walworth JL, Reynolds F (1995) Bioremediation of a petroleum-contaminated soil: Effects of phosphorous, nitrogen and temperature. – Journal of Soil Contamination 4: 299-310.

Wammer KH, Peters CA (2005) Polycyclic aromatic hydrocarbon biodegradation rates: A structure-based study. – Environmental Science and Technology 39(8): 2571-2578.

Wang C, L X, Zhang C (2017) Environmental security control of resource utilization of shale gas drilling cuttings containing heavy metals. – Environmental Science and Pollution Research 24: 21973–21983.

Watanabe T (2002) Pictorial Atlas of Soil and Seed Fungi: Morphologies of Cultured Fungi and Key to Species, 2nd ed. – CRC Press Boca Raton.

Widdel F, Rabus R (2001) Anaerobic biodegradation of saturated and aromatic hydrocarbons. – Current Opinion in Biotechnology 12(3): 259-276.

www.cwciraqpetroleum.com.

www.ifc.org/ehsguidelines