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Characterization and potentials of microbial community in soil and water contaminated with crude oil in Egypt

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Abstract

The present investigation was carried out to characterize the microbial population's inhabitants in a crude oil-contaminated area located in the city of Ras Gharib at the Red Sea, Egypt. Soil, well water, sea water and oil-water-emulsion samples were collected. The samples contained a microbial population not exceeding 1.6 x 10^4cfu g^{-1} or ml^{-1}. The numbers of the indigenous microflora increased noticeably when the samples were incubated in the presence of additional nutrients. A diverse microbial population including different bacterial and fungal genera and species capable for degrading crude oil was proved. A total of 18 bacterial strains and 4 fungal strains capable of growing with crude oil as a carbon source was selected among the cultures isolated (total 614 bacteria and 31 fungi) from different samples. The identities of candidate genera capable of growing with crude oil were confirmed by the 16S rRNA (for bacteria) and 18S rRNA (for fungi). The bacterial isolates were affiliated to five genera of *Pseudomonas*, *Alcaligenes*, *Bacillus*, *Dietza* and *Streptomyces*. The four fungal isolates were affiliated to Aspergillus. *Bacillus subtilis* RGB6 emerged as the top bacterial degrader (68.3%). *Aspergillus terreus* RGF3 and *Aspergillus terrus* RGF1 exhibited the maximum degradation rates of 86.5 and 70.3%, respectively.

Key words: Indigenous oil-degrading microorganisms; crude oil; biodegradation; 16S rRNA; gravimetric analysis

1. Introduction

Petroleum hydrocarbons consist of 50-80% of crude oil and become a greater potential source of contaminants in the soil and water environments. Different hydrocarbon degrading microorganisms have been found in hydrocarbons contaminated environments [1]. Indigenous populations of microorganisms, which are ubiquitous in soil and ground water and self-adapted to hard conditions, actually grow by using the carbon from the pollutants as energy source and cells building blocks. The structure of the microbial community in an environment, influences deeply the degree of oil hydrocarbons degradation. Therefore, in order to characterize and to monitor the native microbial community, the dynamics and the functional potential of both bacteria and fungi in polluted ecosystems is essential for the development of a successful bioremediation strategy [2]. Many methods used for removal and control of crude oil hydrocarbon pollutants include physicochemical and biological treatment. Bioremediation provides the most cost-effective and eco-friendly strategies for the remediation of crude oil contaminated soil and water to bring back its native environment [3]. Understanding the changes in the microbial diversity resulting from contamination of soil and water with crude oil can be useful for the selection of the most effective hydrocarbon degrading microorganisms for crude oil remediation in what is called bioaugmentation.

Autochthonous bioaugmentation, based on the re-inoculation in polluted sites of indigenous microorganisms previously enriched under laboratory conditions, enhanced the microbial activities, thus improving the degradation of hydrocarbons [4]. In order to provide an inoculum for bioaugmentation, the isolation of microorganisms in pure culture from these contaminated environments is fundamental. Although identifications and characterizations of the microorganisms involved in the degrading processes are available [5], less is known on the biodiversity and dynamics of the native hydrocarbons-degrading microbial community of a contaminated soil,

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especially during the enrichment process applied to isolate the most effective strains [6]. The development of effective bioremediation strategies requires an extensive understanding of the resident microorganisms of these habitats. Recent techniques such as high-throughput sequencing (HTS) have greatly facilitated the advancement of microbial ecological studies in oil-polluted sites.

The objective of our study was to evaluate the abundance of indigenous microorganisms in an area with long-term impacts from contamination with crude oil. We have reported on the isolation and characterization of the individuals of this microflora and its potential to degrade crude oil. Another objective was to obtain a collection of hydrocarbons degrading fungi and bacteria potentially exploitable for soil and water bioremediation.

2. Material and methods

2.1. Chemicals

The crude oil used in this study was obtained from a local crude oil refining company with a specific gravity of 0.845 g/cm3 at 25^{o}C.

2.2. Collection of samples

Soil, well water, sea water and oil-water-emulsion samples were collected from an area situated near the city of Ras Gharib on the Red Sea, Egypt (28°21'35.4"N 33°04'36.5"E). The sampling contaminated area had been naturally exposed to oil pollutions since a long time. A total of 5 different samples was collected aseptically over a period of 20 months from the same point between December 2017 and July 2019 as follows: (1) One soil sample (RS) of 0.5 kg was collected at 20 cm depth in pre-sterilized labeled self-sealed plastic bag. (2) Two water samples from crude oil well (B and J) and one water sample from surface sea water (KY) contaminated with oil were collected in sterilized glass bottles. (3) One oil-water-emulsion sample (A) from crude oil well was obtained in sterilized glass bottle. All samples were kept in an icebox and transported to the laboratory then maintained at 4^{o}C until analysis.

2.3. Selective isolation of crude oil-utilizing microorganisms from samples

Bacteria were isolated by means of the enrichment culture technique [7,8]. Either 20 g of soil, 20 ml of well water or 20 ml of sea water samples was transferred to 500 ml flask containing 180 ml of Mineral broth medium (MM) [9] which was composed of (g $_{L}^{-1}$): $Na_2HPO_4.12H_2O$ 9; KH_2PO_4 1.5; NH_4Cl 1.5; $MgSO_4.7H_2O$ 0.2; $CaCl_2.2H_2O$ 0.02; ferric ammonium citrate 0.001 and 1 ml of trace element solution was added to 1 L of the medium. The composition of the trace element solution was (mg $_{L}^{-1}$): $ZnSO_4.7H_2O$ 100; $MnCl_2.2H_2O$ 30; H_3BO_3 300; $CoCl_2.6H_2O$ 200; $CuCl_2.2H_2O$ 10; $NiCl_2.6H_2O$ 20; $Na_2MoO_4.2H_2O$ 30 and Na_2SeO_3 20. The flasks were incubated at 30 ^{o}C on a rotary shaker at 110 rpm to allow the microorganisms to enrich. The resulting enriched culture (at 7 days intervals up to 35 days) was further diluted through streaking on MM agar plates containing 1% crude oil as a carbon source for selective isolation of crude oil degraders. A flame sterilized loop was used for streaking the culture. Plates were incubated at 30 ^{o}C for 5–7 days until appearance of colonies. Individual colonies were picked up and purified by repeated streaking on MM agar containing crude oil. Fungal colonies were collected from the Rose Bengal plates and purified on potato dextrose agar (PDA) [10] at least three times. The PDA medium was composed of (g $_{L}^{-1}$): dextrose, 10; $MgSO_4$, 0.5; chloramphenicol, 0.1; Rose Bengal, 0.05 and agar, 15 [11]. The ability of the purified bacterial and fungal isolates to degrade the crude oil was assessed as described below.

2.4. Preliminary screening of bacterial and fungal isolates for crude oil utilization

Bacterial and fungal isolates were screened for their ability to grow in crude oil by sub-culturing on MM agar that was laced with 1% crude oil and incubated at 30 °C for 3-7 days [8]. Pure bacterial colonies obtained were stored on nutrient agar slants at 4 °C and in Luria-Bertani (LB) medium supplemented with 20% glycerol at -20 °C for further screening and characterization. Fungal isolates were stored on potato dextrose agar (PDA) slants at 4 °C.

2.5. Efficacy of crude oil removal by selected isolates

Fifty ml of the minimal Bushnell Haas medium (BH) [12] were transferred to 250 ml conical flask and 0.5 g (1%) of crude oil as a carbon source was added. The flasks were inoculated with 2 ml of 12 hr grown microbial inoculum of each isolate. The

medium was composed of (g L^{-1}): $MgSO_4$ 0.2; $CaCl_2$ 0.02; KH_2PO_4 1.0; K_2HPO_4 1.0; NH_4NO_3 1.0 and $FeCl_3$ 0.05. The flasks were incubated at 30 °C on a Rotary shaker incubator (Bioblock scientific, model: SI-100) at 180 rpm for up to 25d. The remaining un-degraded crude oil in each culture broth was determined via preliminary gravimetric assay as described below. Un-inoculated control flasks were prepared to detect losses due to abiotic processes.

2.6. Chemical determinations

The crude oil concentration at the beginning of each experiment and the oil remaining after biodegradation was determined gravimetrically as described by Panda et al. [13]. The pH and salinity values were determined with a pH/EC meter (Hanna USA). Moisture contents were determined at 105 °C.

2.7. Molecular identification of bacterial and fungal isolates

Selected bacterial isolates exhibiting a high growth with crude oil as a sole source of carbon were subjected to molecular characterization as follows:

2.7.1. Bacterial DNA extraction from isolates

Bacterial isolates were grown in liquid mineral salt medium supplemented with 1% crude oil (v/v) (9) for 72 h at 30°C then harvested by centrifugation at 12000 g for 5 min. After washing the bacterial pellets for three times using 0.85% NaCl solution, the genomic DNA was extracted using Gene JET Genomic DNA purification Kit (Thermo scientific, Lithuania) [14]. DNA yields and purity were checked using both Nanodrop spectrophotometer and agarose gel electrophoresis.

2.7.2. Bacterial fingerprints and genotypic diversity

The BOX-PCR fingerprints of bacteria were generated for 18 isolates according to Rademaker and De Bruijn [15] using BOXA1R primer (CTACGGCAAGGCGACGCTGACG). Eight µl of the PCR products were separated by 1.5% agarose gel electrophoresis in 0.5 X TBE-buffer for 4 hours (50 V). The BOX-PCR fingerprints patterns were checked and compared visually.

2.7.3. Identification of crude oil degrading bacteria and fungi using 16S rRNA and ITS gene sequencing

The 16S rRNA gene fragments of 9 crude oil degrading bacterial isolates were amplified using the universal primers F-27 (5′-AGAGTTTGATCMTGGCTCAG-3′) and R1494 (5′-CTACGGYTACCTTGTTACGAC-3′) according to Lane [16] and Turner et al. [17] using PCR machine (Bio-rad T100 thermal cycler). For fungal isolates, the internal transcribed spacer (ITS) region was amplified using the universal primers ITS1 (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) according to White *et al.* [18] and Gardes and Bruns [19]. The PCR products were checked via agarose gel electrophoresis then sequenced by Macrogen-Koria.

2.7.4. Phylogenic analysis of bacterial and fungal isolates

The evolutionary history was inferred using the Neighbor-Joining method. The bacterial phylogenetic tree was computed using the Maximum Composite Likelihood method. The analysis involved 52 nucleotide sequences of which 9 sequences of 16S rRNA gene amplified from bacterial isolates of current study while 33 sequences representing the most similar hits were obtained from the NCBI GenBank data base. The fungal phylogenetic tree involved 26 sequences of which 4 sequences of ITS regions amplified from fungal isolates of current study while 22 sequences obtained from the NCBI GenBank data base. Evolutionary analyses were conducted using MEGA5 software.

3. Results and Discussion

3.1. Physicochemical characteristics of samples

After receiving in the laboratory, the soil, water, and oil-water-emulsion samples were analyzed for pH, moisture, carbon, nitrogen, phosphorous, salinity as well as their content of microbiome (Table, 1). The aim of this investigation was to explore the microbiological and physicochemical characteristics of the collected samples. Since petroleum hydrocarbon-degrading bacteria were known to be evolved as a result of existing in close proximity to naturally occurring petroleum hydrocarbons in the environment, the abundance of the indigenous microflora inhabiting the collected environmental samples were evaluated as well.

No viable cell counts could be detected in either oil-water-emulsion or well water B114. However, other samples exhibited viable cell counts of only 1.5×10^2 cfu ml^{-1} (well water J), 1.1×10^4 cfu g^{-1} (soil RS) and 1.6×10^4 cfu ml^{-1} (sea water KY) Table 1. The oil-utilizing microorganisms could be detected in only 2 samples (sea water KY and soil RS) with counts representing only 4.4 and 0.91 %, respectively of the total indigenous community. The observed counts in the present study might represent the

predominated microflora inhabiting the contaminated area. It is known from the published research, that prior exposure to hydrocarbons results in adaptation of the microbial community to utilize hydrocarbons as carbon and energy sources. Bacteria and fungi are known to be the principal agents of hydrocarbon biodegradation. It is reported that the introduction of oily wastes into soil caused appreciable increases in the numbers of both groups [20]. Bhadauria (1997) [21] collected agricultural soil samples irrigated with petroleum refinery effluent, where the bacterial counts ranged between 66 and 8.6×10^{8} cfu $^{g-1}$. Although soil counts cannot be used for analysis of biodegradability of the spilled hydrocarbons, the diversity, and the number of microorganisms at a given site may help to characterize that site with respect to the toxicity of these hydrocarbons to the microbiota, age of the spill and concentration of the pollutant. Fresh spills and/or high levels of contaminants often kills or inhibit a large sector of the soil microbiota, whereas soils with lower levels of aged contamination show a greater number and diversity of microorganisms [22].

In the present study, there was a great divergence in pH between samples from different locations (Table, 1). The sea water KY sample had a pH value greater than 8. The pH was 4.0 in oil-water-emulsion (A) sample. Other samples (soil RS, well water J and well water B114) exhibited pH around 7.

3.2. Molecular identification of bacterial and fungal isolates

Oil-degrading microorganisms are ubiquitous in the environment, particularly in the oil-polluted sites. Both fungi and bacteria have been found to be useful in biodegradation process, even though many researches have been concerned on bacteria in the recent times. Although a wide phylogenetic diversity of microorganisms is capable of aerobic degradation of contaminants, *Pseudomonas* species and closely related organisms have been the most extensively studied owing to their ability to degrade many different contaminants [23]. Of 645 bacterial and fungal isolates obtained in the present study, 18 of them possessing heavy growth with oil were chosen and subjected to the molecular characterization. The phylogenetic analysis of 16S rRNA was performed as follows:

3.2.1. Bacterial fingerprints and genotypic diversity

BOX-PCR fingerprints were generated for 18 bacterial isolates obtained from soil, well water and sea water contaminated samples. The fingerprint profiles (Figure 1) showed the genotypic diversity of tested isolates the only identical fingerprint profiles were detected between the isolates (RBG10, RBG11 and RGB2) (RGB5 and RGB12) (RGB13, RGB14, RGB15 and RGB7) while the rest of isolates showed a unique fingerprint profiles. One representative isolate from each different fingerprint profile was identified based on the sequence of 16S rRNA gene.

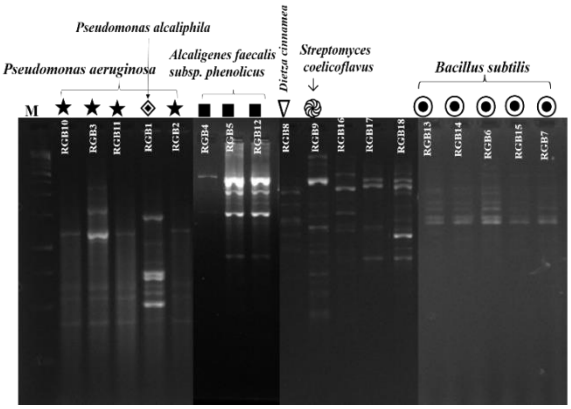

Fig. 1. BOX-PCR fingerprints of 18 bacterial isolates obtained from soil, water and sea water contaminated samples M, 1Kb ladder

3.2.2. Identification of bacterial isolates by 16S rRNA gene sequencing

The 16S rRNA gene sequence analysis of 18 bacterial isolates, which represent different BOX-PCR fingerprint profiles was performed and the high phylogenetic relatedness was confirmed in the neighbor joining phylogenetic tree Figure 2. The 16S rRNA sequence of 2 bacterial isolates (RBG2 and RBG3) showed 99.89% similarity to *Pseudomonas aeruginosa*, isolates RBG6 and RBG7 was 100% similar to *Bacillus subtilis*, while isolates RBG4 and RBG5 showed 99.49%-100% similarity to *Alcaligenes faecalis* subsp. *phenolicus*. Isolate RBG1 was 99.88% similar to *Pseudomonas alcaliphila*, isolate RBG8 was 99.89% similar to *Dietza cinnamea* and isolate RBG9 was 100% similar to *Streptomyces coelicoflavus*. 16S rRNA sequences were deposited in the GenBank under the accession numbers from MW508905 to MW508913 (Table, 2).

3.2.3. Identification of fungal isolates by ITS sequencing

The ITS sequence of 2 fungal isolates (RGF-1and RGF-3) showed 100% similarity to *Aspergillus terreus*, isolate RFG-2 was 100% similar to *Aspergillus sydowii*, while isolate RGF-4 showed 100% similarity to *Aspergillus niger*. ITS sequences were deposited in the GenBank under the accession numbers from MW509090 to MW509093 (Table, 3). High phylogenetic relatedness was confirmed in the neighbor joining phylogenetic tree generated for fungal isolates (Figure 3).

3.3. Efficacy of crude oil removal by identified strains and determination of oil degradation potential

All examined bacterial strains could degrade the crude oil with degradation capabilities ranged from 25 to 68 % (Figure 4). It is obvious that, a removal efficiency of more than 60% was recorded by 6 bacterial strains, of which *Bacillus subtilis* strain RGB6 was characterized by the highest oil removal rate which reduced the oil by 68.3% (Table 4). This strain had the highest bacterial density of 0.778 (OD_{600}) compared to the other two *Bacillus subtilis* strains RGB7 and RGB14 (Figure, 4), might indicating a positive correlation between the bacterial density and the biodegradation capabilities. This finding can also be seen in case of *Pseudomonas aeruginosa* strains RGB3 and RGB2, where the strain RGB2 possessed both higher bacterial density and biodegradation capability. However, when the highest bacterial density was taken into consideration, the bacterial isolates possessing the highest density (*Pseudomonas alcaliphila* strain RGB1 and isolate RGB17; OD600 1.43 and 1.24, respectively) did not surpass other isolates regarding the biodegradation efficiency. Interestingly, these results show that a positive correlation between bacterial density and biodegradation efficiency can be observed mainly within the species of the same genus. Nominal oil degradation (2.3 %) was obtained in the abiotic control flasks.

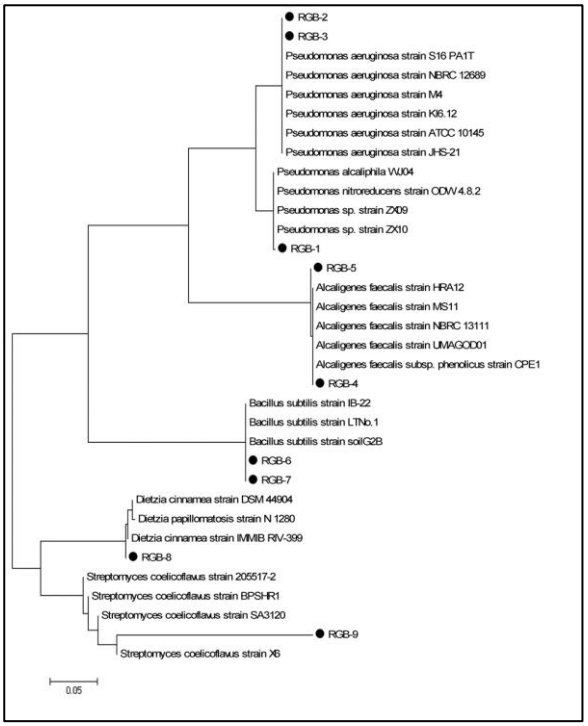

Fig. 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences of 9 bacterial isolates (Dark circles) with the closest hits obtained from the NCBI

Although *Bacillus subtilis* strain RGB6 had the highest oil degradation capability, a slight transition from stationary phase to death phase was observed early at the 5th day until the 7th day. This might suggest that the biodegradation capability of this strain is more likely due to the type of bio-surfactants or enzymes produced. *Bacillus subtilis* is able to produce bio-surfactant species belonging to the surfactin lipopeptide group during its stationary phase of growth [24]. These high molecular weight biosurfactants help stabilize emulsion so that the degradation process is more stable [25]. Bacterial strains such as *Pseudomonas aeruginosa* and *Bacillus subtilis* identified in the present study were also recognized in many ecological studies e.g., Al-Saleh *et al.* [26]; Al-Wasify and Hamed [27] among other hydrocarbon degrading microorganisms.

During incubation, emulsification of crude oil was evident in the culture media in the test flasks, suggesting the production of extracellular bio-surfactant/bio-emulsifier, which may be one of the mechanisms used by these strains to utilize crude oil. The isolates emulsified the crude oil within only 25 days of incubation; on standing the flask, a thin layer of oil was separated out which again became dispersed on gentle shaking. In contrast, the oil layer in control flasks remained on the surface even after 25 days. In published studies, the strains *Ochrobactrum anthropi* HM-1, *Citrobacter freundii* HM-2 and *Ochrobactrum* sp. C1 were found to tolerate high used oil concentration together with emulsification ability of the culture broth medium. [28, 29]

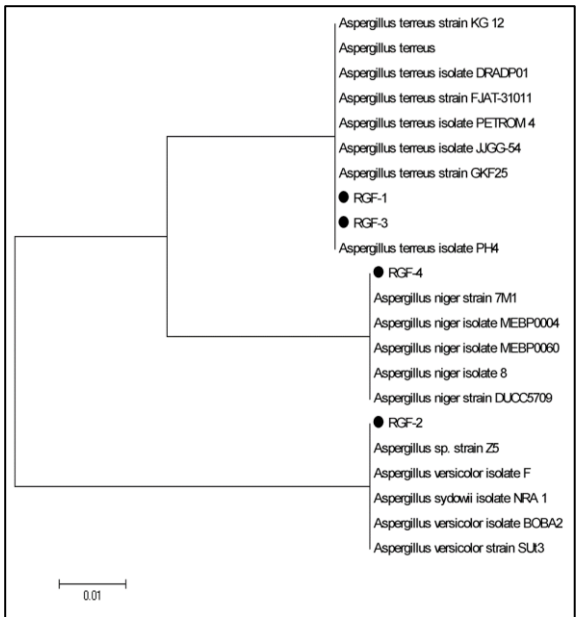

Fig. 3. Neighbour-joining phylogenetic tree based on ITS regions of 4 fungal isolates (Dark circles) with the closest hits obtained from the NCBI GenBank

The high biodegradation efficiency (62.9%) recorded by *P.aeruginosa* RGB2 in our study is comparable to those found by Liu *et al*. [30] who reported that *P. aeruginosa* XJ16 showed the highest biodegradative potential for n-alkanes, with total biodegradation ratio of 98.2% in 10 days. Among five bacterial strains obtained by Mirdamadian *et al*. [31], *Pseudomona*s strain L best degraded (72%) the crude oil in 20 days. Berg *et al*. [32] isolated *Pseudomonas aeruginosa* UG2 from soil contaminated with oily wastes which was found to produce highly emulsifying activity when grown on various substrates. The capability of *Bacillus subtilis* strain RGB6 in reducing the crude oil concentration of 68.3% in our study was superior compared to those described by Ijah and Ukpe [33] who measured 50.4% decrease in crude oil by *Bacillus* strain 61B after 20 days.

Compared to the bacterial strains, the isolated fungal strains possessed higher biodegradation rates since the oil removal efficiencies ranged from 65.3 to 86.5% after 25d of incubation Figure 4. When compared to other isolates, *Aspergillus terreus* RGF3 and *Aspergillus terrus* RGF1 exhibited the maximum degradation rates of 86.5 and 70.3%, respectively (Table 5). Noticeably, there was no clear positive correlation between the fungal density and the degradation capabilities as was observed within the species of the same genus of bacterial strains. *Aspergillus sydowii* RGF2, for example, had almost the highest fungal density over the other 3 *Aspergillus* strains while it possessed the lowest biodegradation capability of only 65.3% after 25 d incubation compared to 67.3, 70.3 and 86.5% with the other *Aspergillus* species. The observed variability of the biodegradation capabilities among the different fungal strains was probably due to the difference in fungal density of each fungus attaining a maximum growth peak and declining after some time, probably as a result of exhaustion of nutrients and release of toxic materials into the medium.

The recorded fungal biodegradation efficiencies in the present study are obviously higher than those reported by Al-Hawash *et al.* [34] who recorded crude oil biodegradation capabilities of 57 and 55% after 14 days by 2 fungal strains, e.g., *Penicillium* strains RMA1 and RMA2, respectively. Moreover, Al-Hawash *et al.* [35] reported a crude oil removal efficiency of 60.3% using *Aspergillus sp*. RFC-1 on the 7th day of incubation which was lower compared to our isolated fungal strains. Furthermore, the recorded high biodegradation efficiency of *Aspergillus terrus* RGF3 of 86.5% in the present study was superior when compared to those described by Al-Nasrawi [36]. The authors isolated 16 fungal strains from sand samples contaminated with oil spill, from which 4 strains were confirmed for biodegradation ability of crude oil with *Aspergillus niger* recorded the highest crude oil weight loss of 8.6%, *Penicillium documbens* (7.9 %) and *Cochliobolus lutanus* (4.7%) whereas the lowest weight loss was demonstrated by *Fusarium solani* strain 421502 (1.9%). *Aspergillus flavus* NIOSN-SK56S22 isolated by *Barnes* et al. [37] from Arabian Sea sediments indicated 62% degradation of crude oil.

When the removal efficiencies are calculated per liter, the consumed crude oil by bacterial strains *Pseudomonas aeruginosa* and *Bacillus subtilis* was fluctuated between 6.04 to 7.196 and 4.264 to 6.844 g L^{-1}, respectively (Table, 4). These efficiencies were obviously higher than those reported by Al-Wasify and Hamed [27], who reported consumed amounts of 1.8 and 1.6 g L^{-1} for both organisms after 28 days, respectively. However, both organisms identified by these authors showed higher degradation percentages reaching 78.86 and 77.84% after 21 days, respectively.

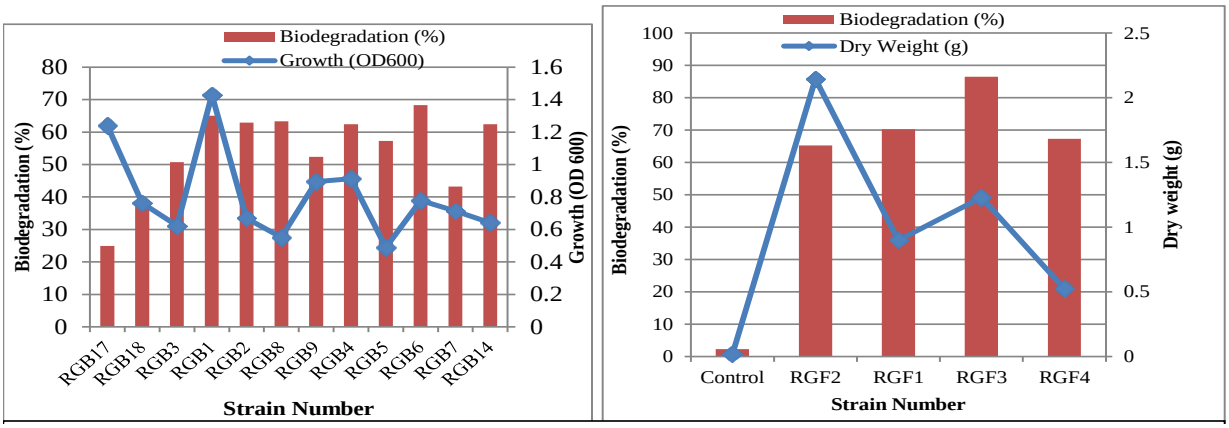

Fig .4. Growth densities and biodegradation potentials of the selected bacterial (left) and fungal (right) strains

On the other hand, calculating the removal efficiencies per liter and day in the present study revealed specific degradation rates between 0.103 to 0.316 g oil L^{-1} d^{-1} for bacterial isolates and 0.288 to 0.36 g oil L^{-1} d^{-1} for fungal isolates (Table, 5). The Gram-negative short rod bacterium RGB18 and the fungal strain *Aspergillus terreus* RGF3 possessed the highest degradation rates, since they could remove 0.316 and 0.36 g oil L^{-1} d^{-1}, respectively. These biodegradation rates of fungi were comparably higher than those reported by Al-Hawash *et al.* [34] recording 0.04 and 0.039 g d^{-1}.

Although it is widely accepted that bacteria and fungi are primary mediators of hydrocarbon degradation, bacteria have been shown to be more versatile than fungi and therefore may play a greater role during biodegradation of hydrocarbons. The results of our study are in contradiction of these findings, since the fungal isolates possessed higher removal efficiencies compared with those in bacterial isolates. Our findings are in agreement with Thomas [38] who published that fungi have been found to be better degraders of crude oil than traditional bioremediation techniques including bacteria. Also, fungi have an advantage over bacteria as fungal hyphae can penetrate the contaminated soil to reach the hydrocarbons that have spread beyond the top layer of the soil [39]. Furthermore, fungi have a higher tolerance to the toxicity of hydrocarbons due to their physiology and adaptation to such variations in the environment and also the presence of mechanisms for the elimination of spilled oil from the environment. Liu *et al*. [40] observed that, at the early stage of remediation, the bacterial community was responsible for the degradation of the saturated and partially aromatic hydrocarbons; the fungal community instead became dominant in decomposing the polar hydrocarbons fraction in post-remediation. Generally, thanks to the variety of extracellular enzymes and fungal hyphae, fungi are the first key players in degrading available contaminants and recalcitrant polymers [41]. Fungal mobilization and degradation of contaminants contribute to release bioavailable intermediates on which, in a later stage, the bacterial community can act more easily [42].

Microbial isolates from the soils that are historically exposed to hydrocarbon pollution exhibit a higher potential of biodegradation than others with no history of such exposure. For that matter, in a crude oil-polluted soil, the biodiversity and microbial prevalence of certain microbe(s) may indicate how well the soil is supporting the growth of that microbe(s) [43]. Fresh spills and/or high levels of pollutants often kill or inhibit large sectors of the soil microbial population, whereas soils with lower levels or old pollution show greater numbers and diversity of microorganisms [44]. It is well known from the published reports that some bacteria use limited carbon sources with a preference for crude oil hydrocarbons and are thus "professional hydrocarbonoclastic" bacteria. For example, *Alcanivoraxs* strains grow on n-alkanes and branched alkanes but cannot use any sugars or amino acids as carbon sources [45].

4. Conclusion

Isolation, identification, and characterization of crude oil utilizing bacteria and fungi was performed. We demonstrated that the oil-polluted soil and water contained a diverse oil-degrading microflora. Potential crude oil degraders were isolated from oil-contaminated area. The capability of these degraders of using crude oil for their carbon and energy requirements could be of further use in the bioaugmentation remediation process for crude oil contamination. The 16S rRNA analysis revealed 9 bacterial strains and 4 fungal strains capable of degrading harmful pollutants like crude oil spills. Fungal degradation can be considered as a key component in the clean-up strategy for petroleum hydrocarbon remediation.

Table 1. physicochemical characteristics and major microbial communities in oil contaminated water and soil samples

	Samples				
Samples Code	**Soil RS**	**Sea water KY**	**Well water 1 J**	**Well water 2 B114**	**Oil-water-emulsion A**
Collection date	2 Oct 2019	12 Jul 2019	2 Feb 2019	15 Jan 2018	11 Dec 2017
Source	Well	Sea	well	well	well
Description	black, with oil smell	mixed with oil	mixed with floating oil	mixed with floating oil	liquid black, homogenized
pH (1/10)	7.6	8.32	6.66	6.6	4.0
Moisture (%)	16.75	-	-	-	-
Carbon (%)	-	-	-	-	57.8
Nitrogen (%)	-	-	-	-	0.82
Phosphorous (ppm)	-	-	-	-	-
Salinity (EC)	185	79.13	-	-	-
Total heterotrophic counts*	1.1×10^4	1.6×10^4	1.5×10^2	Nd	Nd
Oil degrading counts*	1.0×10^2	7.4×10^2	Nd	-	-
Total fungi *	Nd	Nd	Nd	Nd	Nd

*CFU g-1 soil or ml-1 water/oil; Nd, not detected in the exanimated sample; -, not examined.

Table 2. Bacterial identification and accession numbers of 9 bacterial strains

Strain code	Name of strain	Similarity %	Accession number
RGB1	*Pseudomonas alcaliphila*	99.88	MW508905
RGB2	*Pseudomonas aeruginosa*	99.89	MW508906
RGB3	*Pseudomonas aeruginosa*	99.88	MW508907
RGB4	*Alcaligenes faecalis* subsp. *Phenolicus*	100	MW508908
RGB5	*Alcaligenes faecalis* subsp. *Phenolicus*	99.49	MW508909
RGB6	*Bacillus subtilis*	100	MW508910
RGB7	*Bacillus subtilis*	100	MW508911
RGB8	*Dietza cinnamea*	99.89	MW508912
RGB9	*Streptomyces coelicoflavus*	100	MW508913

Table 3. Fungal identification and accession number of fungal strains

Strain code	Name of strain	Similarity %	Accession number
RGF-1	*Aspergillus terreus*	100	MW509090
RGF-2	*Aspergillus sydowii*	100	MW509091
RGF-3	*Aspergillus terreus*	100	MW509092
RGF-4	*Aspergillus niger*	100	MW509093

Table 4. Biodegradation efficiency of bacterial strains towards crude oil

Source of isolation	Identification Code	Isolate/Strain	Maximum growth density (OD)	Oil removal efficiency			
				g oil removed /50 ml after 25 d	g oil removed /L after 25 d	g oil removed L^{-1} d^{-1}	Oil removed %
-	-	Control		0.0122	0.244	0.01	2.3
Soil	RGB17	Gram ve- short rode	1.24	0.1291	2.582	0.103	24.9
Soil	RGB18	Gram ve- short rode	0.763	0.3946	7.892	0.316	37.2
Sea water	RGB3	*Pseudomonas aeruginosa*	0.623	0.3598	7.196	0.288	50.7
Soil	RGB1	*Pseudomonas alcaliphila*	1.429	0.3343	6.686	0.267	65
Soil	RGB2	*Pseudomonas aeruginosa*	0.67	0.302	6.04	0.242	62.9
Soil	RGB8	*Dietza cinnamea*	0.551	0.3436	6.872	0.275	63.3
Soil	RGB9	*Streptomyces coelicoflavus*	0.895	0.3338	6.676	0.267	52.4
Soil	RGB4	*Alcaligenes faecalis* subsp. *phenolicus*	0.913	0.3109	6.218	0.249	62.4
Soil	RGB5	*Alcaligenes faecalis* subsp. *phenolicus*	0.489	0.2757	5.514	0.221	57.3
Well water	RGB6	*Bacillus subtilis*	0.778	0.3422	6.844	0.274	68.3
Well water	RGB7	*Bacillus subtilis*	0.713	0.2132	4.264	0.171	43.2
Soil	RGB14	*Bacillus subtilis*	0.642	0.3158	6.316	0.253	62.4

Table (5): Biodegradation efficiency of fungal strains towards crude oil

Source of isolation	Identification code	Strain	Maximum measured dry weight (g L^{-1}) *	Oil removal efficiency		
				g oil removed/50 ml after 25d	Removal%	g oil removed L^{-1} d^{-1}
-	-	Control	-	0.0122	2.3	0.01
Well water	RGF2	*Aspergillus sydowii*	2.145	0.3651	65.3	0.292
Well water	RGF1	*Aspergillus terreus*	0.9	0.3599	70.3	0.288
Well water	RGF3	*Aspergillus terreus*	1.23	0.45	86.5	0.36
Well water	RGF4	*Aspergillus niger*	0.525	0.3656	67.3	0.293

***Maximum dry weight estimated after different times.**

5. Conflicts of interest

"There are no conflicts to declare"

6. References

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