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A PHYLOGENETIC STUDY OF OIL DEGRADING BACTERIA ISOLATED FROM SELECTED STATIONS IN BASRAH CITY

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Abstract – The target of this work was to isolate and identify indigenous bacteria from local oil-contaminated sites associated with petroleum industries, then studythe relationship among the oil degrading bacteria as well as to the identification of the dominant bacterial community in these areas. In the current study the enrichment culture technique was employed using basal mineral salts medium (MSM) broth supported with crude oil as a sole source of carbon to isolate the desired microorganisms. Depending on the Biochemical and Molecular criteria as well as the genes encoding 16S rRNA, 17 isolates have been identified and characterized according to the genotypic approaches. The 16S rRNA encoding gene was used because its conservative during generations. The successfully identified genera were *Acinetobacter radioresistens, Pseudomonas oryzihabitans, Aeromonashydrophila, Pseudomonas putida, Micrococcus luteus, Pseudomonas aeruginosa, Pseudomonas guariconensis, Achromobacter xylosoxidans, Enterobacter* sp., *Klebsiella* sp., *Staphylococcus* sp., *Bacillus foraminis, Exiguobacterium* sp., *Bacillus firmus, Brevibacillus brevis, Brevibacillus brevis, Stenotrophomonas* sp., *Pseudomonas stutzeri.* The percentage of similarity was very closely related [over 99% of all reported valid genera except *Bacillus foraminis* (96.77) and *Pseudomonas stutzeri* (93.88%)] and all analysis sequences were having Expected (E) value 0.0, which means the matches were significant.

INTRODUCTION

Basrah, the 3rd largest city in Iraq with a total area of (19.070) km². The oil companies activity in Iraq is mainly located in the Basrah city (Republic of Iraq, 2016). The growth of the oil industry, together with a population explosion and the lack of environmental regulations, has caused substantial damage to the local environment (Jaafar, 2017).

Crude oil features makes it an important and effective pollutants of the ecosystem and cause significant damages to humans. Chemical, physical and biological methods were used to treat oil spills, but the critical one of these is the biological by means of bioremediation (Olukunle and Boboye, 2012).

Phylogenetic studies are based on the 16S rRNA gene because it is highly conserved among bacteria and archaea species during generations. Moreover, the use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker. To amplify the 16s rRNA gene which is providing the phylogenetic information, universal PCR primers were used for this purpose. The progression of the techniques for 16S rRNA gene sequence analysis in a natural sample has gently enhanced our ability to detect and identify bacteria in nature (Azizan *et al.*, 2018).

The molecular phylogeny application related to environmental samples has led to the discovery of previously unrecognized microorganisms. However, In various habitat there are many differences between the isolated and naturally occurring microorganisms (Pace, 1997). In the biological sciences, the trees are so important such as systematic and comparative phylogenetic (Solomon *et al.*, 2005).

Depending on the similarities and differences in physical and/or genetic traits, the phylogenetic tree for different biological species is constructed. The joined taxa in the tree are descended from the same

ancestor and each node is mention to taxonomic unit and generally the hypothetical taxonomic units refer to the internal nodes (Azizan *et al.,* 2005).

This study was conducted to identify the evolutionary relationships among indigenous microorganisms in Basrah city that are presumably capable of degrading oil. Day by day the microbial community at Basrah oil contaminated environment is expected to be changed from time to time due to great human activities which related to oil industries.

MATERIALS AND METHOD

Soil sampling sites

Approximately 500kg (0-15cm depth) of soil samples were collected from five different oil contaminatedstations as shown inthe Figure 1 and Table 1. Sample collection bags were used to collect the samples and transported to the laboratory and stored at 4°C, before that, physiochemical parameters (pH and salinity) of the soil samples were checked.

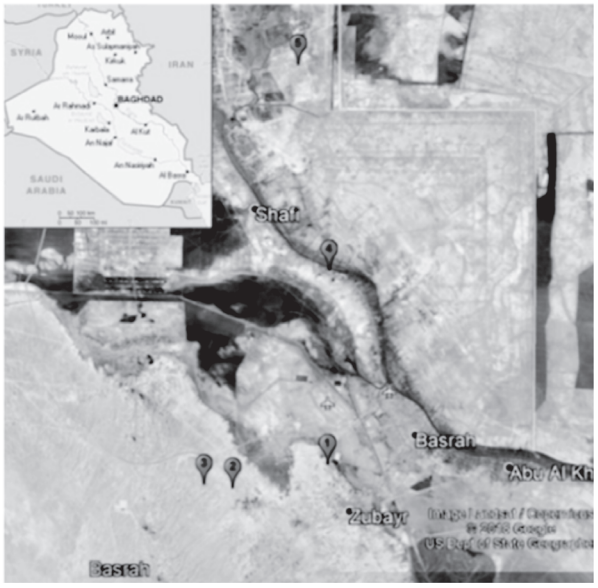

Fig. 1. Sampling stations: 1: Second refining unit, Shuaiba refinery/ 2: Southern Rumaila/ 3: Al-Toba and Al-Nakhaila/4: Nurhan Omar/5:Al Qurna

Table 1. Coordination of sampling stations[7]

No.	Latitude	Longitude
1	30°27′17.35"N	47°39′42.00"E
2	30°25′6.53"N	47°29′35.71"E
3	30°25′24.70"N	47°26′32.30"E
4	30°45′5.91"N	47°39′52.86"E
5	31° 3′58.28"N	

Isolation of bacterial species

One gram of polluted soil was diluted with sterile D.W to 10^{-3} then cultivated in 250 mL conical flask containing 100 mL of basal mineral salts medium (MSM):KCl(0.3g/L), K_2HPO_4(1.03g/L), KH_2PO_4(0.53g/L), $FeSO_4.7H_2O$(0.013g/L), NaCl(1.5g/L), $MnSO4.7H_2O$(0.53g/L), $CaCl_2$(0.23g/L).

2 mL of trace element stock solution composed of (g/L)

$FeCl_3.6H_2O$(0.08 g/L), $ZnSO_4.H2O$(0.75 g/L), $COCl_2.6H_2O$(0.08 g/L), $CuSO_4.5H_2O$(0.075 g/L), $MgSO_4$. H_2O (0.75 g/L), H_3BO_3(0.15 g/L), Na_2MoO_4. $2H_2O$(0.05 g/L), 0.1 ml of sterilized crude oil (provided from Majnoon oil field) was addedas the only carbon source, incubation period was 7 days at $30^{R^{\circ}}$C in the shaker incubator at 150 rpm and the initial pH of medium was adjusted at 8.0. The optical density (OD 600nm) and pH of the liquid cultures were observed at determined time intervals as biodegradation indices.

After the end of the incubation period, one mL of growth culture was diluted with normal saline to 10^{-3}, nutrient agar medium was used to the cultivation of the diluted growth culture at 30 °C for 24 hrs. Then single colonies have been picked from the agar to obtain pure colonies for next further work.

Biochemical and Molecular identification and characterization

Pure colonies of bacteria were cultivated for 24hr, then the following morphological and biochemical criteria were performed (Gram's staining, cell shape, catalase test and oxidase test), DNA was extracted and purified according to the instructions of the company (Qiagen/USA). The 16S rRNA genes were amplified by polymerase chain reaction PCR using a specific set of primers 27F-5′AGAGTTTGATCCTGGCTCAG′3 1492R-5′GGTTACCTTGTTACGACTT′3 to amplify a 1,450 bp fragment of the bacterial 16S rRNA gene in 25 µL mixtures of Bioneer master mix, 2 µl of purified DNA (50 ng/µL), 3 µL of forward and reverse primer (62.5 µmol/L) and d. H_2O was used to completed the volume to 50 µL. Thermocycler (3Prime, UK) with the following thermal profile: A gene amplifier was used to incubate reaction through an initial denaturation at 96 °C for 3 min., followed by 27 cycles of amplification, denaturation at 96 °C for 30 s, primer annealing at 56 °C for 25 sec., primer extension was at 72 ºC for 15 sec. and finally at 72 °C for 10 min. Concentration of 1.5% w/

v agarose gel and 100 bpDNA ladder with 1x of TBE buffer were used to examined PCR amplification product for 40 min at 120mA and 65V, then ethidium bromide solution was used to stained the gel. The amplified nucleic acid was visualized by Computerized UV transilluminator (SYNGENE-GBOX F3,UK).

The PCR products were sequenced and the data base queries were used to estimate the degree of similarity with the sequence of other genes by the NCBI web site.

Computational Analysis of DNA Sequences

After sequencing, the 16S rRNA sequences results of the bacteria were revised then aligned with the NCBI data bankto compare with the other resemble bacteria. Geneious Prime 2019 software version 1.1 was usedto detect the sequences similarity. The most similar sequences were retrieved for each sample. Phylogenetic tree was constructed after the sequences were aligned and trimmed so that the processing has been done with the same length. Multiple Sequence Alignment and evolutionary relationship was determined by comparing the DNA sequences obtained from the sequencing step with the BLAST results obtained from NCBI. These relationships were constructed and the tree was drawn using (Neighbor-Joining method) applied in Geneious Prime 2019 software version 1.1 which downloaded from the official web site.

RESULTS AND DISCUSSION

Physiochemical parameters of the soil samples

The results of physiochemical parameters of collected soil samples were detected and listed in the Table 2, salinity and pH of collected soil samples were detected in order to stimulate the environmental parameters to be dependent on the preparation of the culture media.

Table 2. Physiochemical parameter of soil samples

Sampling stations	pH	Salinity (g/L)
1	8.5	1.4
2	8.4	1.3
3	8.5	1.3
4	8.6	1.6
5	8.6	1.4

Soil samples were collected from different contaminated areas with various petroleum derivatives to obtain different bacterial types, as the enzyme systems found in the oil degrading bacteria differ with different available substrates, and on this basis, the diversity of soil contaminated sources leads to the diversity of different bacterial species.

MSM media was essentially prepared from mineral salts and trace elements, the medium was supported with a crude oil as a sole source of energy which is supplied the bacteria with the organic carbon, predominant growing bacteria in this medium were the oil degrading bacteria, while the others didn't grow because it were unable to utilize the available hydrocarbons source.

Morphological and biochemical criteria

Before the genetic identification, some morphological and biochemical traits of bacteria isolated from contaminated soils must be determined. Table 3 illustrates some of the necessary and important tests that need to be performed to identify the bacterial affiliation to the Gram positive or negative group for the purpose of completing the subsequent genetic diagnosis.

Relation between the growth curve and the degradation activity of the isolates

The ability of Bacterial samples for hydrocarbons biodegradation was tested using crude oil. All samples proved their ability to degrade oil by dispersing the oil layers in the conical flasks in different levels and increasing the turbidity of the MSM medium gradually during incubation periodwhich reflects the growth of bacteria depending on the crude oil as a carbon source Figure 2, More broth turbidity compared with control means more bacterial growth (Aneja, 2007) and that indicated the bacterial bioremediation efficiency in the environment which is in agreement with the findings of and this result was confirmed by monitoring the optical density of the culture media during 7 days of the incubation times as

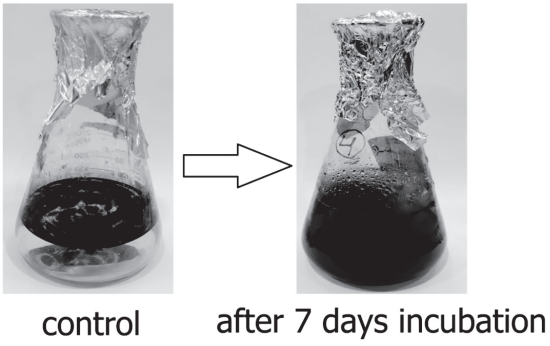

Fig. 2. Oil layer dispersion in conical flasks

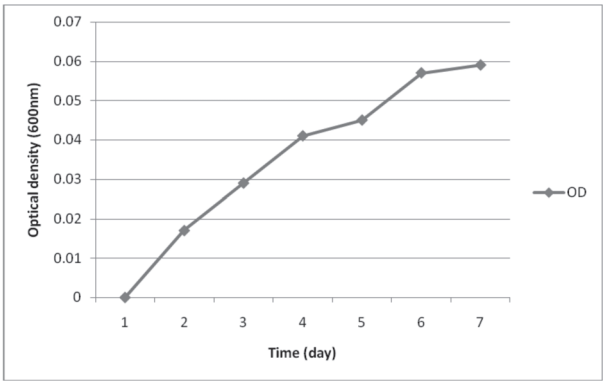

Fig. 3. Optical density of crude oil-MSM medium during incubation period

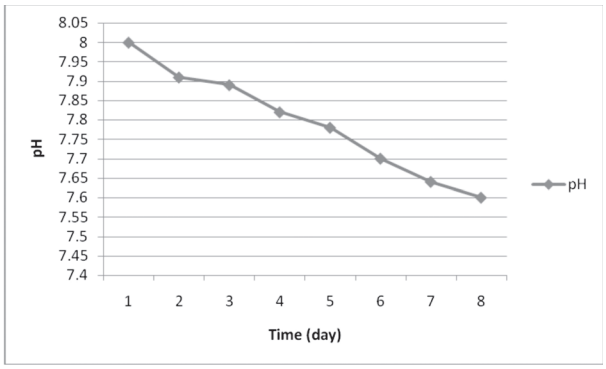

Fig. 4. pH of crude oil-MSM medium during incubation period

shown in the (Figure 3), the OD level was increased by the time (Ojo, 2006).

Effect of degradation on pH

The initial pH of medium was 8.0 at the start of the experiment. During the incubation period, the chemical parameters of the crude oil hydrocarbons was changed due to the action of microbial enzymes, that was the reason of decrement in the pH levels of the isolation flasks within this period as shown in the (Figure 4), also the biodegradation of the hydrocarbons leads to the formation of organic metabolic compounds which caused the reduction in pH, this result agree with (Sepahi *et al.*, 2008).

Computational Analysis of DNA Sequences

The prime goal of the current study is screening for microorganisms adapted in the hydrocarbon polluted soil, and used the petroleum as a source for energy, and can thus be used as potential species for bioremediation of petroleum contaminated soil breaking down hydrocarbons into carbon dioxide and water (Sei and Fathepure, 2009; Zhao *et al.*, 2008). To achieve this goal Seventeen genera were isolated and characterized as *Acinetobacter radioresistens, Pseudomonas oryzihabitans, Aeromonashydrophila, Pseudomonas putida, Micrococcus luteus, Pseudomonas aeruginosa, Pseudomonas guariconensis, Achromobacter xylosoxidans, Enterobacter* sp., *Klebsiella* sp., *Staphylococcus* sp., *Bacillus foraminis, Exiguobacterium* sp., *Bacillus firmus, Brevibacillus brevis, Brevibacillus brevis, Stenotrophomonas* sp., *Pseudomonas stutzeri.* Based on these overview results, the microbial

Table 3. Biochemical test results

Codes of purified bacterium	Gram's stain	Cell shape	Catalase test	Oxidase test
1	-ve	Coccobacillus	+	-
2	-ve	Rod	+	-
3	-ve	Rod	+	+
4	-ve	Rod	+	+
5	+ve	Micrococcus	+	-
6	-ve	Rod	+	+
7	-ve	Rod	+	+
8	-ve	Rod	+	+
9	-ve	Rod	+	-
10	-ve	Rod	+	-
11	+ve	Coccus	+	-
12	+ve	Rod	-	-
13	+ve	Rod	+	-
14	+ve	Rod	+	-
15	+ve	Rod	+	-
16	-ve	Rod	+	-
17	-ve	Rod	+	+

population in that area was more diverse than expected, and our results were nearly similar to the reported results of isolated hydrocarbons degrading bacteria, *including Pseudomonas aeruginosa, Pseudomonasstutzeri, Enterobacter cloacae, Stenothrophomonas maltophilia*, *Bacillus cereus* and *Bacillus pumilus* **(**Borah and Yadav, 2014; Zafra *et al.,* 2016).

Subsequently the sequences results of 16S rRNA genes of our study are aligned with the corresponding sequences of recorded hydrocarbon-degrading organisms, and the resulting phylogenetic tree which revealed that these isolates were clustered into *Pseudomonas* spp. (five isolates) and *Bacillus* spp. (two isolates) as shown in Figure 6. In this figure, it is obviously that *Pseudomonas* spp. is the most dominant genera among the isolates (~ 30%), that is may be due to its own different enzymes and metabolic pathways required to degrade components of crude oil and that agreed with (Das and Chandran, 2011). Moreover, *Pseudomonas* sp. was also able to use different hydrocarbons as carbon sources and energy and it is well known that *Pseudomonas* among the bacteria with high remediation potential of different types of hydrocarbons (Stamenov *et al.,* 2015).

Stamenov on the other hand, the similarity percentage was very closely related [over 99% to the all reported valid genera except *Bacillus foraminis* (96.77) and *Pseudomonas stutzeri* (93.88%)]. FASTA sequences for 17 samples obtained from DNA sequencing were used to identify the species through BLAST. All sequences retrieved were having Expected (E) value 0.0, which means the matches were significant. The genera were then identified and listed as their accession number and identity percentage in Table 4. The trees scale bar represents a 0.02% difference in nucleotide sequences and it's obviously from the tree that the isolates clustered with their specific matches from the database.

The choice of method for tree reconstruction depending on the size and the quality of the dataset. In this project, Neighbour-Joining (NJ) of constructing phylogenetic trees were applied Figure

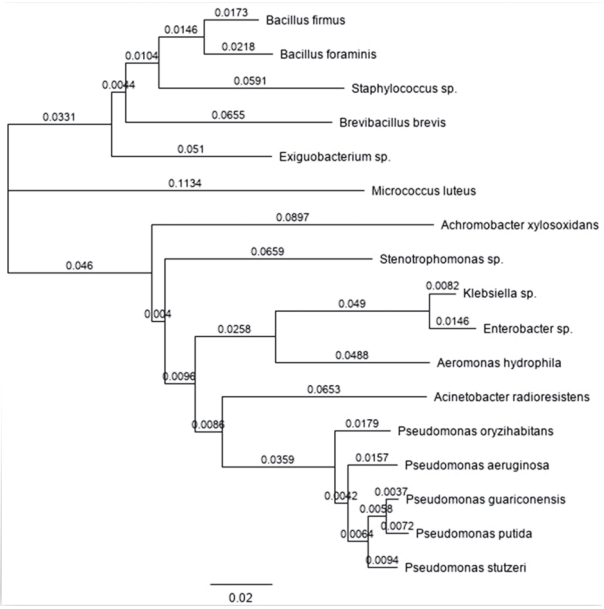

Fig. 5. A phylogenetic tree created by Geneious Prime 2019 version 1.1

Table 4. Accession no. and identity percentage of 16S rRNA genes of isolated bacteria

Sample code	Organism	Accession no.	Maximum identity (%)
1	*Acinetobacter radioresistens*	MK334657.1	99.86%
2	*Pseudomonas oryzihabitans*	MG571765.1	99.93%
3	*Aeromonas hydrophila*	MK007301.1	99.86%
4	*Pseudomonas putida*	MH084903.1	99.93%
5	*Micrococcus luteus*	KY495217.1	99.64%
6	*Pseudomonas aeruginosa*	MK386864.1	99.86%
7	*Pseudomonas guariconensis*	MG674358.1	99.86%
8	*Achromobacter xylosoxidans*	GU014534.1	99.86%
9	*Enterobacter* sp.	EF522821.1	99.79%
10	*Klebsiella* sp.	AY540111.1	99.86%
11	*Staphylococcus* sp.	JN975939.1	99.51%
12	*Bacillus foraminis*	GQ903407.1	96.77%
13	*Exiguobacterium* sp.	KU758893.1	99.86%
14	*Bacillus firmus*	HQ678663.1	99.51%
15	*Brevibacillus brevis*	MK318220.1	99.29%
16	*Stenotrophomonas* sp.	LT724239.1	99.72%
17	*Pseudomonas stutzeri*	KT986148.1	*93.88%*

(5) and the phylogenetic tree pattern is unrooted due to easy detection among the genera.

The bacteria isolated in this study were considered to be suspected hydrocarbon tolerance. They can grow in MSM medium containing 1% crude oil. 17 genera were isolated and characterized as *Acinetobacter radioresistens, Pseudomonas oryzihabitans, Aeromonas hydrophila, Pseudomonas putida, Micrococcus luteus, Pseudomonas aeruginosa, Pseudomonas guariconensis, Achromobacter xylosoxidans, Enterobacter* sp., *Klebsiella* sp., *Staphylococcus* sp., *Bacillus foraminis, Exiguobacterium* sp., *Bacillus firmus, Brevibacillus brevis, Brevibacillus brevis, Stenotrophomonas* sp., *Pseudomonas stutzeri.*

To make the demonstration of the relationship among the identified species more easily all of them have been grouped in a tree then the lineage of the species can be easily identified through the tree constructed. In general, the identification of dominant bacterial communities in oil contaminated soils of Basrah environment contributes significantly to facilitate the task of biologic treatment with minimal cost via green technologies.

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