Detection of 16SRNA Gene variation of bacterial strains isolated from water marsh polluted with oil spills southern of IRAQ

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Abstract
Oil spills closer to Marshes and human populations have greater ecological and economic impacts., especially which result of oil spills occurring of drilling oil wells south of Iraq. In this study, ten samples were collected from different zones of ALHammar marsh. The Samples were symboled with the letter W (W1 to W10). The samples were analyzed with GC to detect types of hydrocarbons found in these samples also COD test we're done to find the values of higher pollution. The result found that sample W6 had the higher volution my measuring total hydrocarbon compound and COD value wow W1 Sambo showed this valuation among all samples. these two samples were chosen to detect types of bacteria. serial dilutions were done to each sample reached 10 5. each dilution was cultured on nutrient agar to observe growth density in each dilution. the result found that the W1 sample contain mixed bacterial growth with a high number of bacterial growths compared with the W6 sample which included less bacterial growth. The W6 samples growth were cultured on blood agar and MacConkey agar in order to detect the type of gram bacteria, bacteria were examined with Vitek 2 to detect strains type, the result showed that the strains were Citrobacter freundii and Bacillus cereus. DNA extraction was done and primers were designated in order to detect fragments of 16SRNA.PCR and electrophoresis were done to detect target fragments which were appeared at 1500pb as product size. Sanger sequences were done for both strains. the results were analyzed with bioinformatic tools genius software The result of Blast nucleotides was showed that new local strains were detected. The accession numbers of two new strains were submitted to GenBank Bacillus cereus IQ2021772 with accession number MZ958640, version MZ958640-1. The second new strain was Citrobacter freundii strain IQ2021771 with accession number MZ958639 and version MZ958639-1. Phylogenetic trees were constructed for both strains, the results of tracked ancestors of these strains found ancestors were isolated from coastal and industrial zones of China.

Keywords Hydrocarbones.16SRNA. Bacillus cereus. Citrobacter freundii

1. Introduction

Oil spilled and waste hydrocarbons has become a major problem of Environmental pollution. The hydrocarbon compounds contaminate water and terrestrial and atmospheric ecosystems. Oil spills are dangerous for a number of reasons: flammability, explosive vapors, toxicity, hydrogen sulphide, and oxygen exclusion. Explosive vapors following oil spills can be displaced by air to nearby cities where they can be easily igniting; emission of hydrogen sulphide gas can affect the airways of residents near spills. In humans, the effects of oil translate into inhibition of protein synthesis and nerve synapse function, impairment of plasma membrane and interruption of membrane transport systems; in fact, hydrocarbons can damage DNA leading to mutagenesis, carcinogenesis, and decreased reproductive capacity [1] oil spills enrich these environments with hydrocarbon clastic bacteria ([2]). Similar to the microbially mediated breakdown of natural organic matter, biodegradation mediated by indigenous microbial communities is the ultimate fate of the majority of oil hydrocarbon that enters the marine environment [3]. It has been observed that the saturated components of crude oil (alkanes and cycloalkanes) and particularly then-alkanes of intermediate lengths (C10^C20) are biodegraded more readily. Alkanes of shorter chain length are more toxic, while those of larger chain length (C20-C40) are hydrophobic solids dicot to degrade due to their low aqueous solubility and bioavailability [4]. Polyaromatic compounds (PAHs), especially those of high molecular mass, are degraded slowly and with low efficiency [4], the utilization of microorganisms to degrade such pollutant has been found to be a promising alternative [5]. Microorganisms have potential to detoxify hazardous organic compounds by means of polymerization, mineralization, or transformation [6]. The bioremediation process depends on the ability of microorganisms to remove the hydrocarbon pollutants completely[7]. Interestingly, numerous hydrocarbon-degrading microorganisms are capable of producing biosurfactants/emulsifiers agents that could increase the solubility of these sparingly or insoluble substrates and facilitate their utilization as sole carbon sources [8]the target of this research is to detect mutations that occurs in indigenous bacteria du to modification and adaptation for oil spills in marshes water after

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isolate and detection bacteria strains[9]. indigenous bacteria such as bacillus sp. and Citrobacter sp. as indigenous bacteria improved persist and modified to survive in an environment polluted with hydrocarbons (Durval et al., 2020).

The 16S rRNA gene is used for phylogenetic studies.[11] 16S rRNA gene can be used as a reliable molecular clock because 16S rRNA sequences from distantly related bacterial lineages are shown to have similar functionalities[12] The mutation rate of an organism is an evolved characteristic and is strongly influenced by the genetics of each organism, in addition to strong influence from the environment.[13]

The study aims to

1-Detect indigenous bacterial strains found in marshes water that have the ability to live and survive in the oil-polluted environment

2- Use bioluminescent assay to confirm the correlation between colony-forming units (CFU) and ATP in terms of relative light unit (RLU)

3- Detect mutations occurs within the 16SRNA gene for strains collected from oil spills water samples of Al Hammar Marshes

4- Construct a phylogenetic tree for strains' lives and survival in the polluted environment as a tool to find and track closer strains of study query.

2. Material and Method

Ten samples were collected from the different zone of ALHammar marshes, the samples were examined with COD and GC mass in order to expertise hydrocarbons concentration and COD values in order to choose the most polluted zone and less one as the study of interest, The samples were symboled with the letter W (from W1 to W10). Heterotrophic Plate Count (HPC) was used to estimate the number of live heterotrophic bacteria that were present in a water sample[14]. A sample of water was put on a plate which contains Nutrients that the bacteria need to survive and grow. Nutrient media was used in order to observe the growth of bacteria within two areas before and after spill area of ALHammar marshes, (Reasoner, 1990). BacTiter-Glo™ Microbial kit was used for this purpose, which was designed for either single-tube or multiwell-plate formats for high-throughput screening (HTS). The assay procedure involves adding a single reagent (BacTiter-Glo™ Reagent) directly to bacterial cells in medium and measuring luminescence (Jing Lu et al., 2014). Blood Agar and MacConkey agar were used to grow strains grow in oil polluted samples that differentiated g-ve bacteria (Violet, 2015)Vitek2 systems use Advanced Colorimetry™ were used to detect strains grow in polluted samples. Genomic DNA was isolated from bacterial growth according to the protocol of ABI Opure for bacteria grow in polluted samples. Primer design were used to detect 16S RNA gene fragment forward and reverse for strains of polluted samples 27F 5`-AGAGTTTGATCCTGGCTCAG-3`, 1492R5`-TACGGTTACCTTGTTACGACTT-3`primers were supplied by Macrogen Company in a lyophilized form. Lyophilized primers were dissolved in a nuclease free water to give a final concentration of 100pmol/µl as a stock solution. A working solution of these primers was prepared by adding 10µl of primer stock solution (stored at freezer -20 C) to 90µl of nuclease free water to obtain a working primer solution of 10pmol/µl. [15] The volume of nuclease free water (µl) 300 Concentration (pmol/µl) for primer 10027F and 1492R. PCR was done to amplify target gene fragment 16 SRNA (7).

Standard Sequencing

PCR product were sent for Sanger sequencing using DNA Analyzer (ABI3730XL), automated DNA sequences, by Macrogen Corporation – Korea. The results were received by email then analyzed using geneious software.

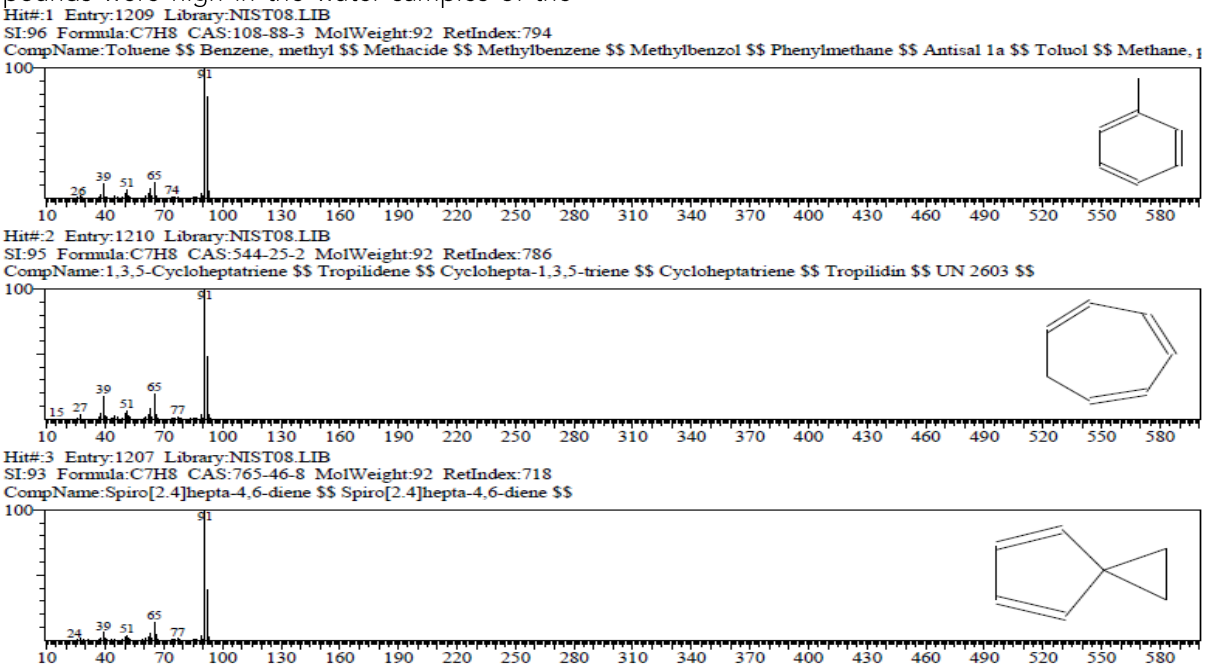
3. Result and Discussion

Ten Samples were collected from different areas of ALMessahab and Salal of AL Hammar marshes. The samples were examined with COD and GC mass in order to experience values of COD and T.C.O concentration. The results were variant from region to another as shown in table (1)., The sample W6 showed higher degree of hydrocarbons pollution, so W6 sample was chosen to detect indigenous bacteria which have the ability to live and survive in such environment. Also, W1 samples which described with low concentration of hydrocarbons T.O.C and COD value was chosen to study bacteria growth which living in the unpolluted environment. The samples were diluted to 10-5 and cultured with nutrient agar. The results of sample W1 which depicted with low concentration of hydrocarbons and COD value showed mixed and high bacterial growth, that may refer to indigenous bacteria found in marshes samples which described with bacterial abundance (YaQin etal,2016)

Table (1). concentration of Total organic compound and COD values for marshes water and soils

Water Sample No.	T.O.C mg/l	COD mg/l	Sediment Sample No.	T.O.C mg/l	COD mg/l
W1	14.1	61	W1	73.2	315
W2	74.4	320	W2	158	683
W3	86.7	373	W3	134	528
W4	82.3	354	W4	213	920
W5	46	198	W5	74.4	320
W6	131.3	565	W6	229	987
W7	92.7	399	W4	97.6	420
W8	121	522			
W9	118	510			
W10	110.9	477			
EPA,2001	50 - >100	100	-	200	-

The presence of these compounds along with many other hydrocarbon compounds in the water and soil samples of Mar Al-Mashab and the general estuary comes from the discharge of partially treated or untreated wastewater or from polluted water runoff from oil field facilities (oil rig platforms, unpolluted oil storage tanks). roads, loading platforms, etc.) to surface watercourses that directly feed the marshes. Where the results of the study showed that the results of the analysis and diagnosis that the total hydrocarbon compounds (Total Hydrocarbon) were high in the water samples of the Marshes of Al-Mashab and the Salal of the Hammar Marsh. The results of the study showed that the results of the analysis and diagnosis that the total hydrocarbon compounds were high in the water samples of the Marshes of Al-Mashab and Al-Salal marshes of the Al-Hammar Marsh, and this appears from the high peaks of the gas spectrum appearing in the figures, especially the high amount of these compounds in the soil samples in the study area. The most important petroleum compounds that were diagnosed in the water and soil samples of the Hammar Marsh are: hydrocarbons, especially poly aromatic hydrocarbons (PAH), benzene, toluene, ethylbenzene and xylene (BTEX) as shown in figure (1). Therefore, they are toxic to humans and environment. The presence of Poly aromatic hydrocarbons (PAH) compounds in such environment induced indigenous bacteria to adaptation for hydrocarbons pollution [10] .



Fig(1).. types of aromatic hydrocarbons found in marshes samples

as shown in table (1) .W1 samples are included many types of bacterial strains .while W6 sample had less growth of bacteria that that refer to the ability of some strains to change in genes ,subsequently enzymes utilize hydrocarbons as source of carbone[4]

Culturing and Bioluminescent results

The number of colony forming unites (CFU) for samples collected from unpolluted area of AL-Hammar marshes were higher than other of polluted area, that may be refer to the ability of adaptation of some indigenous bacteria, that agree with [16]who showed that some indigenous bacteria have an ability to modified to hydrocarbones polluted environment. The results were confirmed by using bioluminescent assay in which cell viability were estimated through ATP amount [17]Table (2). showed (CFU) and amount of ATP for each dilution. The assay was sensitive enough to detect the ATP content of individual cells, therefore the concentration of ATP was directly related to the number of bacteria cells present in a sample [18]

Table (2). values of colony forming units(CFU) and relative light units(RLU) for samples dilutions from stock to 10-5 before polluted and after polluted area of AL Hammer marshes

Sample W1			Sample W6		
dilution	CFU	RLU	dilution	CFU	RLU
stock	>300	14600	stock	250	8419.33
10^{-1}	300	12670	10^{-1}	150	3426.49
10^{-2}	200	8103	10^{-2}	40	3040.43
10^{-3}	30	7860	10^{-3}	20	936.041
10^{-4}	10	4757	10^{-4}	2	560.013
10^{-5}	2	1174	10^{-5}	0	490.011
Blank	0	350.02		0	358.006

The data obtained from water samples were used to construct a graph by plotting values of RLU on the x-axis and values of CFU readings on the y-axis. R2 factor showed high correlation between colony

forming units (CFU)and relative light unit (RLU) which reflect higher sensitivity [18]As shown in figure (2).and figure (3).

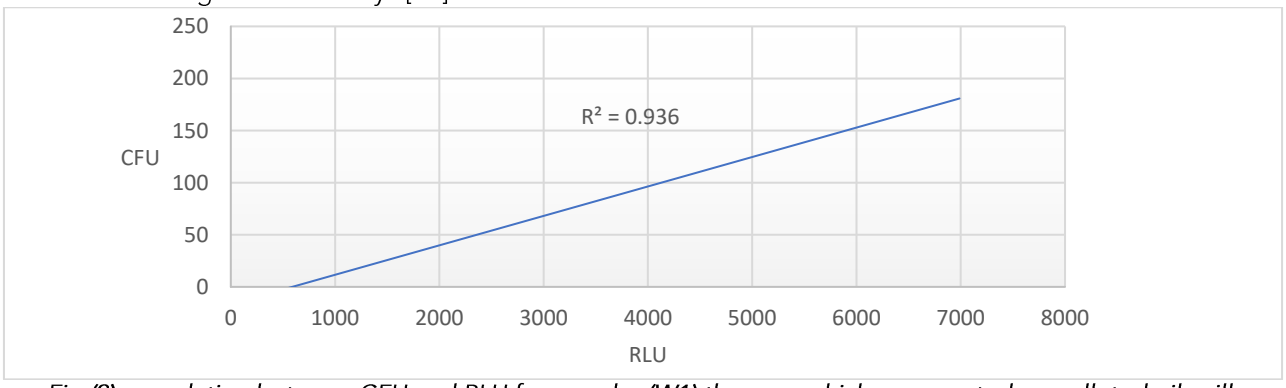


Fig (2). correlation between CFU and RLU for samples (W1) the zone which represented un polluted oil spills area ,the R2 =0.93 showed high correlation between two values

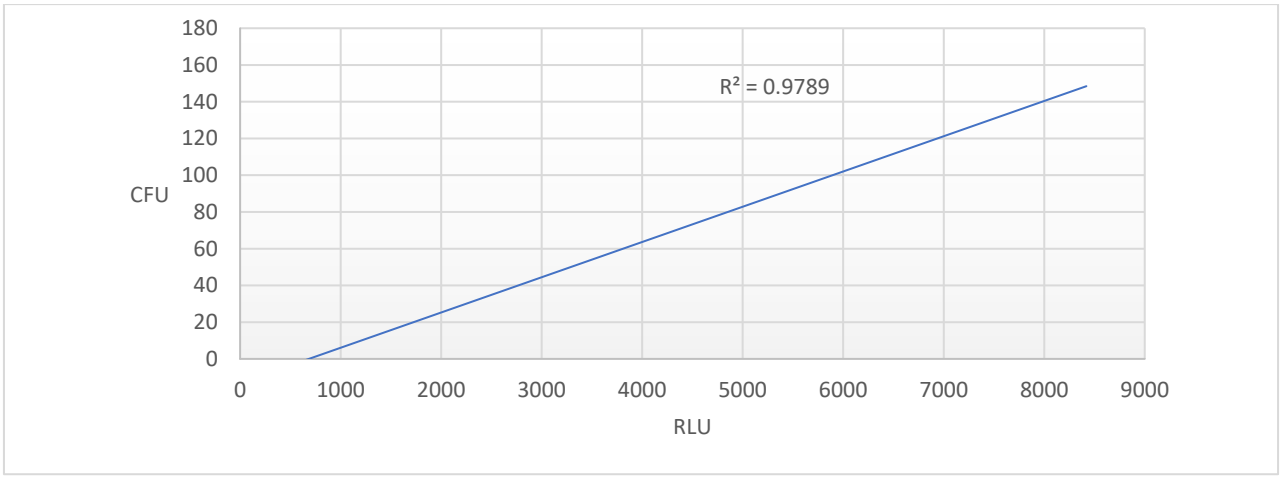


Fig. (3). Correlation between CFU and RLU for samples (W6) oil spills area ,the R2 =0.97 showed high correlation between two values

Detection of resistant strains

Vitek 2 was used for this purpose, the results showed two strains found in samples of oil spills. The strains were included Bacillus cereus and .Citrobacter freundii as shown in table (8) and (9) . These two strains are frequently mentioned in most studies of hydrocarbon pollution [19] .One major advantage of the new VITEK 2 system is its speed in reliably identifying gram-negative rods within 3 h.[20] . Successful growth of all tested on aliphatic and aromatic hydrocarbons suggests that they, like all hydrocarbon clastic microorganisms, produce monooxygenases and dioxygenases (hydroxylases), respectively[14]It is likely that if a system performs well in a stress test (like the VITEK 2 system in conjunction with the ID-GNB card) it will also do so in a weighted laboratory profile 88% Probability Bacillus cereus , therefore, it is predicted that the evaluated system may also perform well under the conditions of a routine clinical laboratory.[20] An accuracy rate of 95% Probability Citrobacter freundii identification to the species level after 3 h (including the strains for which simple, rapid tests had to be performed) .The results of detection founded Bacillus cereus and citrobacter frundii strains in polluted sample that confirm studies which mention the ability of this strains to live and survive in such polluted water. the ability to survive refer to enzymes pathway due to genes mutation that agree withZhang (2020) who mention that there are four superoxidase (SODs) in Bacillus cereus 0–9, and this coexistence of multiple homologous enzymes is of great significance in the evolution of bacteria. also, that agree with Ibrahim, (2016) who found that strains HM-1 and HM-2 of. Citrobacter freundii can be employed to develop a cost-effective method for bioremediation of used engine-oil-polluted soil.

Amplification

Among mixed isolated bacteria, two strains Bacillus cereus (isolates No. A) and. Citrobacter freundii had the greatest ability to treat oil wastewater, they had been subjected for molecular identification by using 16SrRNA gene sequence Applied Biosystems (ABIPRISM 310) at Sigma Company. Based on obtained data, isolate A showed 99 % similarity to members of genus Bacillus and was thus designated as Bacillus sp. and was deposited in GenBank with Accession number MZ958640 and. Citrobacter freundii with Accession number MZ958639.

4. PCR Results

PCR products for both strains were electrophoresed on agarose gel. The 16SRNA fragment genes for both strains were appeared clearly at position 1500 bp the

location at which 16SRNA appeared normally [22] As shown in figure (4).

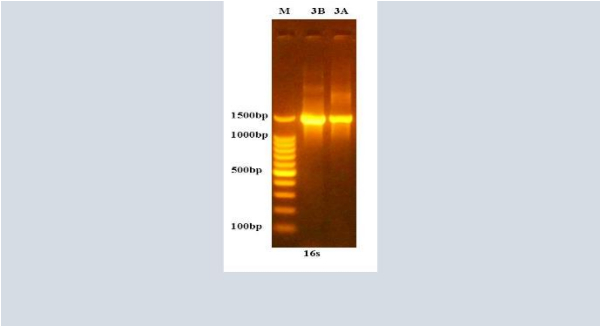


Fig. (4) Results of the amplification of 16s RNA gene of Unknown bacterial species were fractionated on 1% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 1-7 resemble 1500bp PCR products

16SRNA consider as method of bacterial identification [23] .the method was confirmed the Vitek 2 results of bacterial strains Bacillus and Citrobacter,

Bioinformatic and Data Analysis

BLAST results

Isolates were assigned a putative taxonomy using BLAST [22]The observation that long-read sequencing can identify 16S polymorphisms within the same genome has important implications. First, it demonstrates that it is not valid to assume that high-throughput sequence reads differing by one or few nucleotides represent a distinct taxon Within a single genome, two or more 16S sequences may be identical, whereas others may be unique. Sequence data for each isolate were quality filtered and adapters removed as described above. Filtered sequences were reoriented using align. Seqs of. the NCBI databases. alignments of nucleotides of query were done showed variation of about 1% of other strains sequences, subsequently the two strains were recorded within NCBI databases as new strains with accession number those procedures used when variation of sequence higher than 1%. FASTA form was used_ to store full length by size., The most abundant unique sequence for each isolate was then extracted (on the assumption it was the least likely to contain sequencing errors) and was used as a reference against which to align all reads for that isolate. Analyses of sequence alignment showed mutations such as point mutation means changing in single nucleotide, that subsequently change codon of which consist of three nucleotides., this codon responsible of one amino acid., so changing one nucleotide will change gene expression and then protein function. Other variations as shown in table (3). For Bacillus cereus., the Blast analyzed showed deletion in single nucleotide so that shifting codon of gene. Changing in that fragment of gene(16SRNA) for both strains explain the modification and adaptation which occurred for those strains in order to live within hydrocarbons spill environment. that agree with Ibrahim, (2016) who explain the truth of variation within this gene as urgent tool to survive in environment polluted with hydrocarbons spills. poly aromatic hydrocarbons (PHA). were significantly result of polluted analyzed by GC mass, the two strains have the ability to survive in such type of hydrocarbons. Earlier investigators also found that the same bacterial species isolated elsewhere could grow on oil and hydrocarbons as sole sources of carbon and energy, that agree with Zhang. (2020) who mention of grow this truth. So, this study confirms the fact that those type of bacteria have the ability to remediate PHA hydrocarbons and in the same time found new isolates survive in Iraqi marshes within oil polluted water.

Table (3). The variation of query sequence of Citrobacter *freundii* isolated from polluted W6 marshes

	Location no.	Mutation type	Variation
1	1085	Point mutation	C to A
2	8	Deletion	T
3	1411	Deletion	T
4	954	Point	T to C
5	947	Point	C to G
6	972	Point	A to G
7	987	Point	C to T
8	8	Deletion	A
9	216	point	G to C
10	127	POINT	C to A
11	172	POINT	A to T
12	168	POINT	A to G
13	162	POINT	G to C
14	188	POINT	G to A
15	189	POINT	G to C
16	192	POINT	C to T

DNA sequence results were analyzed using NCBI Blast a nucleotide. The results showed similarity of 98% of other restrains. That strains were recorded as a new strain in NCBI site with accession number compared and alignment a query result with other 16 S or any citrine showed variation in 16 S in gene fragment of about point mutation to deletion as showed in table (4) that herniation real events in

history in that adaptation used hydrocarbon as a source of carbon and shoe versus to survive in such poor you to the environment. hydrocarbon-degrading microorganisms are capable of producing biosurfactants/emulsifiers agents that could increase the solubility of these sparingly or insoluble substrates and facilitate their utilization as sole carbon sources[19]

Table (4). sequence variation of query Bacillus cereus Blast result			
	Location no.	Mutation type	variation
1	1423	Deletion	C
2	1428	Point	C to G
3	8	Deletion	A
4	1422	Deletion	C
5	512	Deletion	G

More than 50 sequence s of the same 16SRNA fragment compared together with study sequences(query) of. Citrobacter freundii and Bacillus cereus. each nucleotide alignment with one of the same locations. The variation detects clearly if point mutation or deletion or conversion as shown in figure (8).

Nucleotide sequence accession numbers

The accession numbers were submitted to GenBank for both new strains Bacillus cereus IQ2021772 with accession number MZ958640, version MZ958640-1. The second new strain was Citrobacter freundii strain IQ2021771 with accession number MZ958639 and version MZ958639-1 (www.NCBI.com).

Construction of Phylogenetic Tree

Mega software was used to bult phylogenetic tree. Analyzed of tree structure showed the position of query (target sequence) within other strains, also discover the closer strain of query and also tracking the region at which closer strains were isolate. The phylogenetic tree for Bacillus cereus as shown in figure (5). The tree showed that the ancestor strain of study query was EGQ3 with accession number MZ497322, tracking this strain isolated zone found it was isolated from China (NCBI .com). also as shown in figure (6), the. Citrobacter freundii phylogenetic tree showed the closer strain for the study query was the strain MT94653 with accession number ZTP3, the strain was isolates from India Mumbai. while the ancestor for both strains was the strain MF716709, this strain was isolated from Nanchang Jiangxi of China, when tracking the regions of those isolates find both strains isolated from coastal and industrial zone (NCBI .com). this tracking confirmed with finding [24] that coaster zone considers as source of bacteria community. Phylogenetic comparison of 16S RNA gene sequences from oil-degrading bacterial isolates. and sequences retrieved from oiled (boldface). Only isolates lineages are included in the analysis. The most similar sequences identified by BLAST are indicated by GenBank accession number. The bootstrapped neighbor-joining phylogenetic tree was generated in MEGA using the maximum composite likelihood model with gamma-distributed rates and pairwise deletion. 16S RNA sequences from oil-degrading isolates,

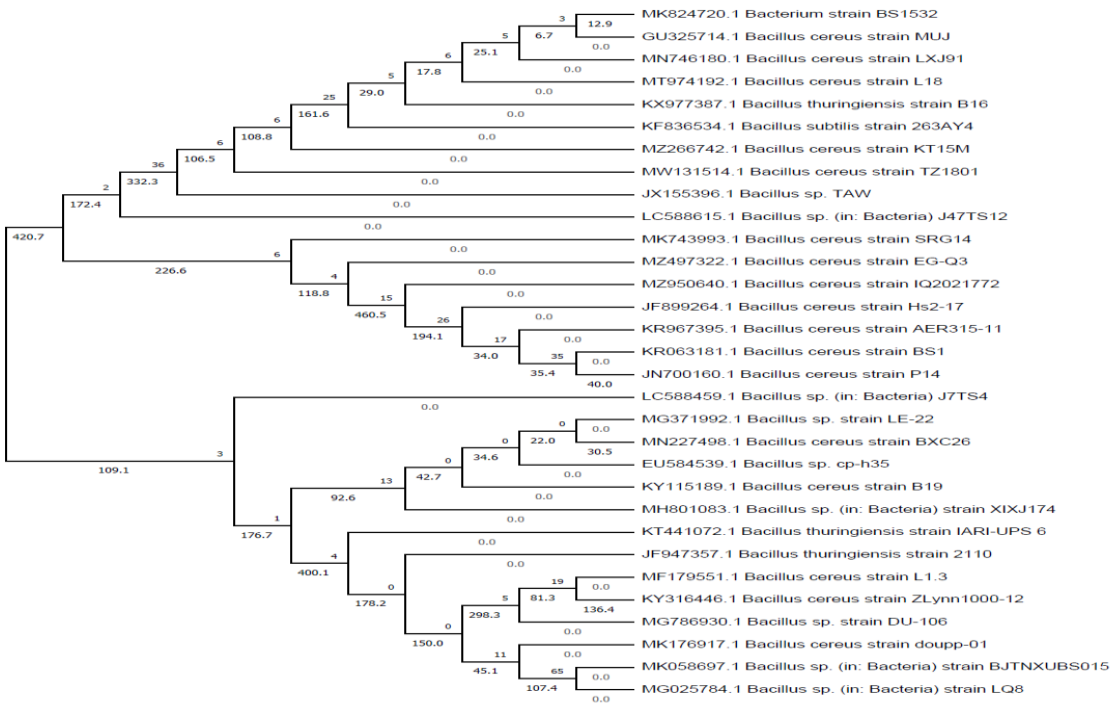


Fig (5)Neighbour-Joining method of phylogenetic tree based on the partial 16S RNA gene sequence, showing the phylogenetic relationships between the Bacillus cereus and the most closely related strains from the GenBank.

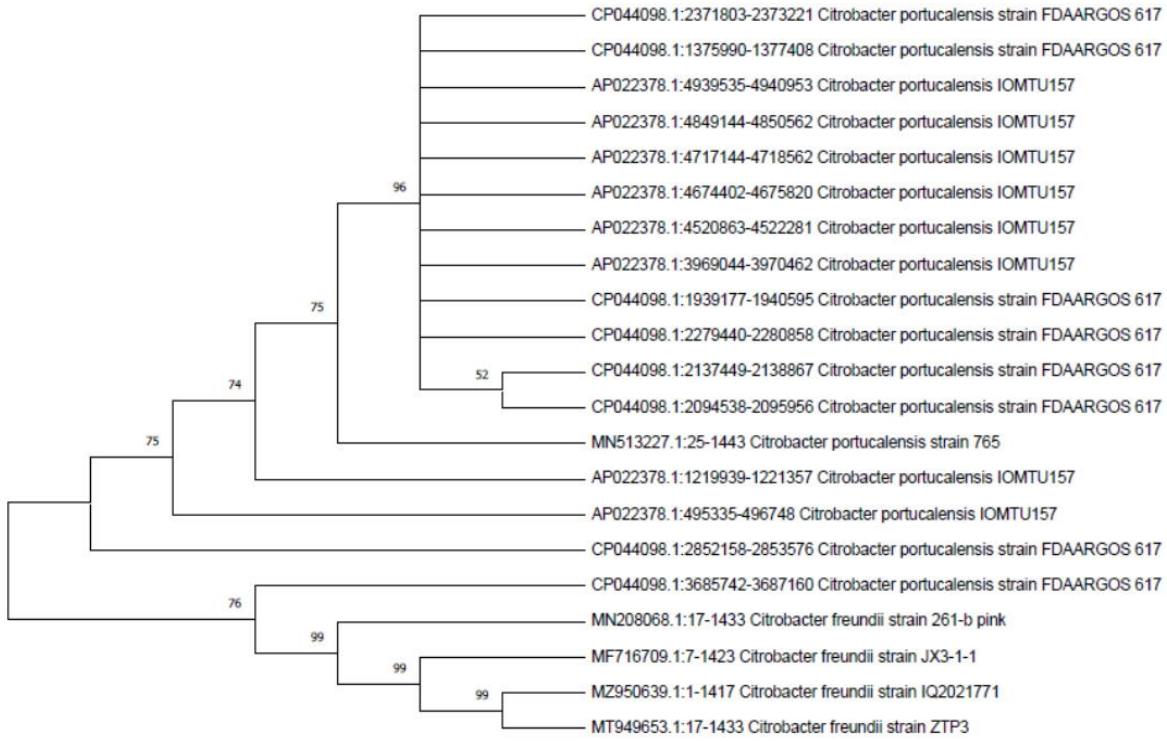


Fig. (6) Neighbour-Joining method of phylogenetic tree based on the partial 16S rDNA gene sequence, showing the phylogenetic relationships between them. Citrobacter freundii and the most closely related strains from the GenBank.

5. Conclusion

The results showed

1-Marshes water polluted with hydrocarbons spilled of drilling oil well with high T.O.C concentration and COD value

2- Hydrocarbons polluted in marshes effected on the indigenous bacteria, subsequently, normal ecosystem structure

3- Citrobacter freundii and Bacillus cereus have the ability to live and survive in environment polluted with hydrocarbons.

4- Two new strains were found in Iraqi water marshes have the ability to live and survive in polluted water

5- phylogenetic tree found the ancestors of new Iraqi strains coming from coastal zone of India and China.

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