



ISOLATION AND IDENTIFICATION OF NEW HALOTOLERANT BACTERIAL STRAINS FROM SALTY FOOD

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ABSTRACT

This study aimed to isolate a halotolerant bacterial strain, four samples were collected from four sources (salty food) during February 2023. four bacterial isolates were obtained using Modified Nutrient Agar (MNA) by raising the concentration of sodium chloride to 5% as a medium for isolation. Bacterial isolates were initially diagnosed based on culture and morphological characteristics. The obtained isolates showed varying sensitivity to five types of antibiotics, including ampicillin, azithromycin, amoxicillin, cefexin, and gentamycin. The isolates also showed varying ability to grow in a temperature range from 10 to 50 °C. The results indicated that the isolates were able to grow differently in different concentrations of sodium chloride, which included (5, 10, 15, 20, 25 and 30%), two isolates showed their ability to grow at a concentration of 30% of sodium chloride. These two isolates were subjected to molecular diagnosis based on the sequences of the 16s rRNA gene, and the result showed that they are two new, previously undiagnosed strains that were registered at the National Center for Biotechnology Information (NCBI), which are *Oceanobacillus* sp. strain *Elham A* and *Oceanobacillus* sp. strain *Thana*.

Key words: halophilic bacteria, high salinity, antibiotic sensitivity, 16SrRNA.

عزل وتشخيص سلالات بكتيرية جديدة متحملة للملوحة العالية من الأغذية المالحة

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الخلاصة

هدفت الدراسة الى عزل سلالات بكتيرية متحملة للملوحة، جمعت اربعة عينات من اربعة مصادر (اغذية مالحة) خلال شهر شباط 2023 لغرض العزل، تم الحصول على اربعة عزلات بكتيرية باستعمال الوسط المغذي الصلب المحور (Modified Nutrient Agar (MNA) وذلك برفع تركيز كلوريد الصوديوم الى 5% وسطاً للعزل، شخصت العزلات البكتيرية بصورة اولية اعتماداً على الخواص المزرعية والمورفولوجية، اظهرت العزلات المستحصل عليها حساسية متفاوتة تجاه خمسة انواع من المضادات الحيوية شملت Ampicillin, Azithromycin, Amoxicillin

*This article is taken from the doctoral dissertation of the first researcher.



50 لغاية 10 من تراوّح مدي حراري من 15, 20, 25, 30 درجة منوية، وتشير النتائج الى قابلية نمو متفاوتة للعزلات في تراكيز مختلفة من كلوريد الصوديوم شملت 15, 20, 25, 30%، واظهرت عزلتان قابليتهما على النمو بتركيز 30% من كلوريد الصوديوم، أخصبت هاتين العزلتين الى التشخيص الجزيئي بالاعتماد على تتابعات الجين 16s rRNA واظهرت النتيجة بانهما سلالتين جديدين غير مشخصتين مسبقا تم تسجيلهما في المركز الوطني لمعلومات التقنيات الحيوية NCBI وهم *Oceanobacillus sp. Strain Thana ElhamA* .

الكلمات المفتاحية: البكتيريا المحبة للملوحة، الملوحة العالية، الحساسية للمضادات الحيوية، 16SrRNA .

INTRODUCTION

Extremophiles are microorganisms that can withstand harsh conditions including (pressure, temperature, salinity and pH), extremophiles come in a variety of forms that correspond to various environmental circumstances (Espliego, *et al.*, 2018). There are many types of extremophiles, each comparing to several conditions in which microorganisms have inhabited. These microorganisms produce many bioactive compounds such as enzymes and antibiotics (Dumorné, *et al.*, 2017). Extremophiles divided into different groups depending on the different conditions of their habitat. Among many extremophiles, halophiles are found in an environment with high concentration of salt. By preserving a balance between the inside and outside of the cell Halophilic microorganism can withstand extremely high salt concentrations and resist osmotic stress (Oren, 2010). Halophiles are increasingly significant in biotechnological applications because of this characteristic. Based on their tolerance to salt halophilic are categorized as extreme (20-25%), moderate (15-20%), and slight (5-10%) (30). According to reports organic nitrogen compounds and pollutants which can be found in food, fertilizers, poisons, organic materials and explosives can be degraded by moderately halophilic bacteria (García, *et al.*, 2004), Furthermore they may be appropriate organisms for the bioremediation of hypersaline environments because they have distinct metabolisms and can withstand high salinities (Le Borgne, *et al.*, 2008).

The cumulative impacts of elevated heavy metals concentrations as a significant effect on the microorganisms that inhabit hypersaline environment, because of this microorganisms that live in these conditions need to be adapted to high salinity and heavy metals (Salman, & Ahmaed, 2019). Because both bacteria and planet cell accumulate a common number of cytoplasmic solutes within conditions of high salinity, there cellular responses with high osmolarity are strikingly like or closely parallel in their mechanisms (Mohamedin, *et al.*, 2018).

It is estimated that there are 8 billion people on the planet by 2050, this is expected to increase roughly 10 billion people worldwide, the demand for food product increased as a result of the sharp increase in global population (Mesa-Marín, *et al.*, 2019). In addition to being used for human needs, water is a vital resource for all living things and is employed in industrial and agricultural projects (Puspaningrum, & Titah, 2020). The world population



growth and technological advancement have been recognized as major factors in the search for improved supplies and a reduction in surface and ground-water pollution, due to these issues researchers are looking into alternative purification technique such as brackish water desalination, which uses salt-water, additionally the removal of dissolved solids generally referred to desalination (**Puspaningrum, & Titah, 2020**). This study aimed to isolate and identify bacterial strains from high salinity foods and study some of their characteristics as a prelude for later use in the treatment of drain water with high salt concentrations and converting it into suitable water for irrigation in the current water scarcity in Iraq.

MATERIALS AND METHODS

Isolation sources

Saline food samples (local production of cucumber brine) were collected after exposing the cans to the air for 48 hrs. by opening the can lids. on February 2023.

Culture media

Modified nutrient agar (M NA)

The MNA medium was prepared as described by the manufacturing company. The media was autoclaved at 121°C and 15 pound/inch² for 15 min. The prepared media was modified by adding 5% NaCl.

Modified nutrient broth (M N B)

The MNB medium was prepared as described by the manufacturing company. The prepared medium was modified by adding 5% NaCl.

Isolation and purification

Decimal dilutions of the samples were prepared and the MNA medium was inoculated with 1 ml of all the decimal dilutions by pouring plates in sterile atmospheres, after that the media was left until solidification and placed to the incubator at 37 °C for 48 hours (2,23). In order to purified the isolates, they were sequentially transferred into modified N.A. by streaking method and the plates were incubated at 37°C for 48hrs. After that ,the one colony of each isolates were transferred to the solid slant medium, and kept at 4 °C for using it in subsequent experiments(**Al-Badran, & Al-Shamary, 2019**).

Cultural and morphological identification of bacterial isolates:

Morphological and Microscopical Characterization of the bacterial isolates were detected by observing the shape, size and color, texture and edge shape of the bacterial colonies, and by microscopic examination of bacterial cells after staining with gram stain and spore stain with malachite green for bacterial cells (**Al-Musawi, & Al-Shamary, 2021, De Vos, & Garrity, 2009**).



Halotolerant test: In order to identify the halotolerant isolates and rank them based on their level of tolerance to deferent concentrations of NaCl, the four isolates were cultivated on N.A media that contained (10, 15, 20, 25, and 30%) NaCl and incubated at 37°C for 48hrs .and the results was observed to detect the Halotolerant isolates.

Antibiotic sensitivity test

Bacterial cultures were prepared using modified NA medium by the streaking method and then adding antibiotic discs at sterile atmospheres, five types of antibiotics were used(Ampicillin, Azithromycin, Amoxicillin, Cefexin, and Gentamycin) as a tablet for laboratory use ,the plates were incubated at 37°C for 24 hours ,then the results of the sensitivity of the bacterial isolates to the antibiotics were observed and recorded(Matuschek, & Kahlmeter, 2014).

Ability to grow at different temperatures test

The selected bacterial isolates cultured on modified N.A. media and incubated at (10,20,30,40 and 50°C) for 48 hours, to examine their ability of growth in different range of temperatures, the results were observed and recorded after the end of the incubation period for each isolate.

Molecular identification

Following ribosomal RNA amplification in bacteria sequence were analyze and microorganism data was confirmed using rRNA data base of(NCBI), every procedure such as the extraction of bacterial DNA, PCR amplification, gene sequencing, and assembly, for Bacteria, PCR on 16S rRNA using 27F and 1492R primers, Table(1) and yielding of 1,300bp or more sequencing data (Fierer, *et al.*, 2005 , Majeed, & Al-Shamary, 2020).

Table (1): Primers used in identification experiment.

Primer Name	Seq.	Annealing Temp.°C	Product Size(bp)
27F	5'-AGAGTTGATCCTGGCTCAG-3'	60	1500
1492R	5'-TACGGTTACCTTGTACGACTT-3'	60	1500

These primers were supplied by the Korean company/ Macrogen in a lyophilized form. Nuclease- free water was used to dissolve lyophilized primers, resulting in a stock solution at a final concentration of 100 pmol/μl. To produce a usable primer solution with 10pmol/μl., 10 μl of primer stock solution which was kept at (-20 °C) mixed with 90μl of nuclease-free water.



DNA Extraction

Genomic DNA was extracted from two bacterial isolates according to the protocol of (ABIOpure).

Quantitation of DNA

In order to assess the quality of samples for use in subsequent processes the concentration of extracted DNA was measured using Quants Fluorometer. For 1 μ l of DNA, 200 μ l of diluted Quantifluor Dye was mixed. After 5min incubation at room temperature, DNA concentration values were detected.

Polymerase chain reaction (PCR):

Table (2): PCR master mixture conditions.

Compounds in the master mix	Vol. (μ l)
Master mix	12.5
10 P mole Forward primer	1
10 P mole Reverse primer	1
Nuclease free water	8.5
DNA extract	2
Total volume	25

A vortex was used to mix the master mixture, Table (2), for a brief period of time. The PCR thermo- cycler was filled with the tube. By the end of the reaction time, the devise was programed in accordance with Table (3), and the amplification of extracted DNA was performed.

Table (3): PCR Program.

Steps	°C	M:S	Cycle
Initial Den.	95	05:00	1
Denaturation	95	00:30	
Annealing	60	00:30	30
Extension	72	01:00	
Final Ext.	72	07:00	1
Hold	10	10:00	

Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to verify the existence of amplification following PCR amplification. Regarding the criteria for extracted DNA, PCR was entirely reliable.

Preparation of Agarose

- Amount of 100 ml of 1X TAE was taken in a flask.
- Agarose 1.5 gm. (for 1.5%) was added to the buffer.



- The solution was heated to boiling (using Microwave) until all the gel particles were dissolved.
- Ethidium Bromide 1 μ l of (10mg/ml) was added to the Agarose.
- The Agarose was stirred in order to get mixed and to avoid bubbles
- The solution was left to cool down at 50-60°C.

Casting of the horizontal agarose gel

After sealing the tow edges of the gel-tray with cellophane tape, the Agarose solution was poured in, and it was allowed to solidify for 30 minutes at room temperature. the gel was put in the gel-tray after the comb was carefully taken out 1XTAE- electrophoreses buffer was added to the tray until it covered the gel surface by 3-5mm.

DNA loading

PCR products were loaded directly. For PCR product, 5 μ l was directly loaded to well. Electrical power was turned on at 100v/m Amp for 60min. DNA moves from Cathode to plus Anode poles. The Ethidium bromide-stained bands in gel were visualized using Gel imaging system.

Standard Sequencing

PCR product was sent for Sanger sequencing using ABI3730XL, automated DNA sequences, by Macrogen Corporation – Korea. Using BLAST nucleotides software, the results have been compared with the information available on the NCBI database in order to identify the selected bacterial strains.

Result and Discussion

Isolation

The process of microorganisms isolation is the cornerstone for obtaining pure isolates for conducting physiological and biochemical tests (Mohamed, & Al-Shamary, 2022). Four bacterial isolates were obtained from Four saline food isolate sources (A, B, C, D) as showed in Table (4). Saline food were chosen as a selective pressure environment on the bacterial isolates to be obtained. The obligate halophilic microorganisms are unable to survive in the environments without high salt concentration due to their macromolecular modifications in the primary and secondary structure of enzymes such as enzyme synthesis becomes dependent on the presence of high salt concentrations. At the same time, there are organisms that live in salty environments, but their enzymes are sensitive to salinity (Espliego, et al., 2018). These organisms depend on increasing the concentration of compatible solvents inside the cell to face the high external osmotic pressure, if this is possible. Solvents protect enzymes from being inhibited by salts by providing them with some aggregates. Some sources indicate that among the adaptations that bacteria use to salinity is that their cell walls do not contain Muramic acid,



and instead there are complex polymers containing sulfur, which makes them resistant to antibiotics, as noted. The proteins and enzymes present in their cell membranes contain negative amino acids, so they need high salt concentrations to stabilize the weak hydrophobic bonds, which are unstable in the presence of water alone (Mesa-Marín, *et al.*, 2019). These proteins and enzymes suffer morphological changes when the salts are removed, which leads to the loss of their function. These changes include: Unraveling their structural folds, it was also observed that the fatty components in the membranes of halotolerant bacteria do not contain phosphate linked to the glycerol as is usual with an ester bond, but rather contain long hydrocarbon chains of Isoprenoids linked to the glycerol with ether bonds, which are more stable in high salt concentrations (Oren, 2010). It was also observed that a modification occurred. In interactions between proteins and fats, a more stable modification occurs at high salt concentrations. It has also been noted that potassium ions are very important for linking the 30S, 50S, and then 70S units, thus carrying out the translation process correctly and binding proteins. (Tuhina, & Swati, 2016).

Table (4): Bacterial Isolation sources and codes.

Isolate	Source
A	Pickled Cucumber with salt
B	Pickled Cucumber with vinegar
C	Pickled Cucumber with salt
D	Pickled Cucumber with salt

Cultural identification: The growing bacterial colonies on the modified medium (N.A+ 5% NaCl) after 48h. of incubation at 37 °C showed that all four isolates had sticky, creamy-white colonies with a dense growth center with branched ends at the edges of the colonies. Table(5).

Morphological identification: Gram staining showed that all isolates had rod cells (bacilli) positive for Gram stain (Figure1) and after using spore staining the bacillus sp. isolates showed that are spore formers (Figure 2).

Table (5): Cultural& microscopic identification characteristics of bacterial isolates.

Isolate	Shape	color	Colony	Gram stain	Spore forming &location
A	bacilli	Creamy-white	Sticky with a dense growth center with branched ends at the edges of the colonies.	+	+Terminal
B	bacilli	=	=	+	+Terminal
C	bacilli	=	=	+	+Terminal
D	bacilli	=	=	+	+Terminal

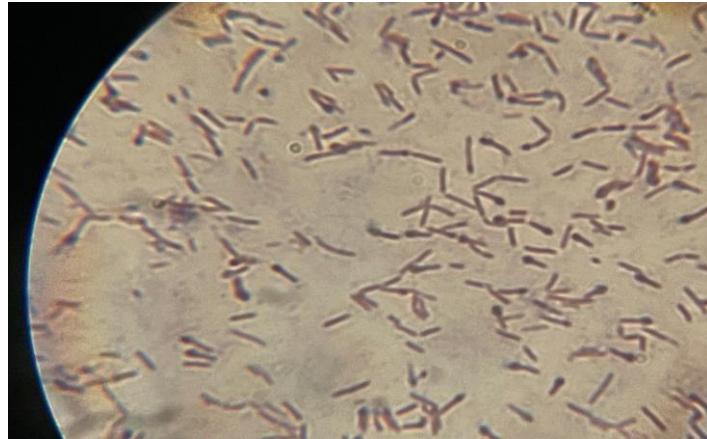


Figure (1): Morphological identification of bacilli bacterial isolates.

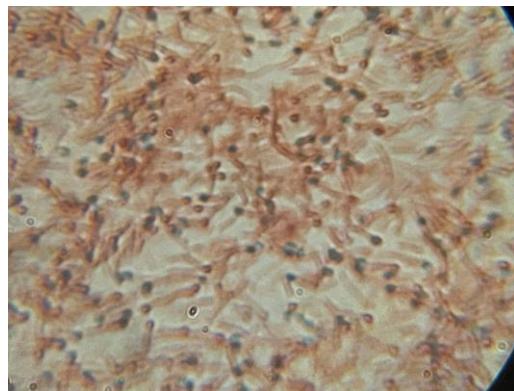


Figure (2): Terminal Spores formed by bacterial strains.

The capability of growing at various temperatures:

All the four bacterial isolates showed varying ability to grow in a range of temperatures Table (5), as the best growth for all isolates was within the range of 30-40 °C, while isolates A and B were able to grow at a higher rate, reaching 50 °C. There is a relationship between the high salinity and the growth temperature of Halophilic and halotolerant bacteria (Schneegurt, 2012). The growth temperature and the optimal growth Temperature. are affected by the NaCl concentration in the growth media (Schneegurt, 2012), so the isolates A1 and B1 which are tolerant to high salt concentration showed a growth at wide range of Temperature (10 - 50°C). Water environments with high salt concentration are usually located in tropical areas with high evaporation rates at high daytime temperatures, in addition to that salty water cooling faster during the night or winter season. The majority of halophilic and halotolerant microorganisms are mesophilic or slightly thermophilic (Hochstein, 2020) .



Table (6): The ability of bacterial isolates to grow at different temperatures.

Isolate code	10°C	20°C	30°C	40°C	50°C
A	++	++	+++	++	+
B	++	++	+++	++	+
C	+	++	+++	++	-
D	+	++	+++	++	-

*(++)heavy growth, (++)moderate growth, (+)weak growth, (-)no growth

Antibiotic sensitivity: Through the results, it was found that the four bacterial isolates are sensitive to different levels towards the types of antibiotics used in the experiment Table (6), which means that the effect of antibiotics is variable according to various factors ,the producing of enzymes such as β -lactamases, alterations of cell wall permeability and chromosomal mutations and activation of efflux pumps are few of the mechanism of drug resistance (Mapara, et al.,2015). Antibiotic resistance and multi-drug resistance are common in halophilic bacteria that have been isolated from their natural habitat as well as pathogenic bacteria, the bacteria can either transform by absorbing naked DNA from surrounding environment or they can transfer plasmid through conjugation (Shinde, & Thombre, 2016).

Antimicrobial resistance in bacteria is frequently linked to the presence of plasmids, and the majority of these extra chromosomal replicates carry drugs resistance genes, mega- plasmids and plasmids have previously been reported in halophiles (Argandoña, et al., 2003). It has previously been documented that plasmids are presents in halophile isolates from tannery wastes (Ghosh, 2010). As the results summarized in Table(7), all the isolates in general are sensitive to Ampicillin and Amoxicillin which are inhibits cell wall synthesis, while the other types of antibiotics have molecular level effects at the bacterial cell like inhibit DNA replication and protein 30s and 50s inhibition .

Table (7): Sensitivity of bacterial isolates to some types of antibiotics.

Isolate	Ampicillin	Azithromycin	Amoxicillin	Cefixime	Gentamicin
A	+	+	+	-	+
B	+	+	+	-	+
C	+	+	+	-	+
D	+	+	+	-	+

(+)*sensitive, (-)not sensitive .

Halotolerant: All four isolates showed their ability to grow intensively in the modified nutrient agar media containing concentrations of sodium chloride between 5-20%, all isolates were not able to grow on media free of sodium chloride, isolates A and B showed their ability



to grow in concentrations that reached 30% of Sodium chloride Table(8). Microorganisms that classified as halophilic need NaCl concentration reach to 12% to grow well (Ventosa, 2006). The main mechanism of salt tolerant operates by internal retention of balancing solute(K+) equal to external NaCl concentration. The second mechanism involves protein with acidic and nonpolar amino acids, the protein needs high salt concentration to balance its charge for its optimal activity (Tuhina, & Swati, 2016).

Table (8): Effect of different NaCl concentrations on the growth of bacterial isolates.

Isolate	NaCl %						
	0	5	10	15	20	25	30
A	-	++	+++	+++	+++	++	+
B	-	++	+++	+++	+++	++	+
C	-	++	+++	+++	++	+	-
D	-	++	+++	+++	++	+	-

(++)*heavy growth, (++)moderate growth, (+)weak growth, (-)no growth

Molecular Identification:

Genetic diagnosis technology is considered one of the latest technologies used in recent years, especially in the field of diagnosis and genetic modifications to benefit from microorganisms in many fields (Auda, & Khalifa, 2019).

Isolates A and B were selected from the previous experiment, as they showed tolerance to the highest concentrations of sodium chloride and were diagnosed genetically. The results of DNA extraction Figure(3), were send to Macrogen/ Korea, detection of nitrogenous base sequences Figure(4), and matching them with the NCBI International Gene Bank database showed that the two strains are new, named and registered in the NCBI World GenBank database under the code (OQ978219) and (OQ825943.1) Oceanobacillus sp. Strain Elham A and Oceanobacillus sp. Thana. figure (5).

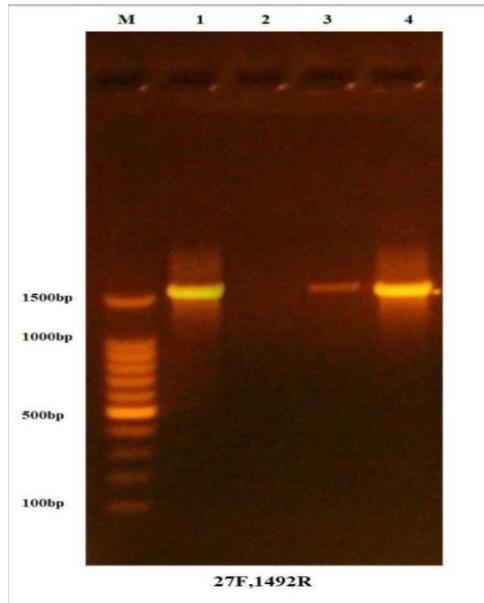


Figure (3): The 16s RNA gene amplification results of undefined bacterial species were separated on 1.5% Agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes resemble 1500bp PCR products.

Sample 1: GAGCGCGGGGA AGCGAACCGGA ACTCTTCGGA GGGAAAGTTCG TGGAACGAGC GGCGGACGGG
 61 TGAGTAACAC GTAGGCAACC TGCCTGTAAG ACTGGGATAA CTCGCGGAAA CGCGAGCTAA
 121 TACCGGATAA CACTTTCCAT CTCCTGATGG AAAGTTGAAA GGCGGCTTT GCTGTCACTT
 181 ACAGATGGGC CTGCGGCGCA CTAGCTAGTT GGTGAGGTTAA CGGCTCACCA AGGCGACGAT
 241 GCGTAGCCGA CCTGAGAGGG TGATCGGCCA CACTGGGACT GAGACACGGC CCAGACTCCT
 301 ACGGGAGGCA GCAGTAGGGGA ATCTTCCGCA ATGGACGAAA GTCTGACGGA GCAACGCCGC
 361 GTGAGTGATG AAGGTTTCG GATCGTAAA CTCTGTTGTC AGGGAAGAAC AAGTACGATA
 421 GTAATCTGATC GTACCTTGAC GGTACCCGAC CAGAAAGCCA CGGCTAACTA CGTGCCAGCA
 481 GCCGCGGTAA TACGTAGGTG GCAAGCGTTG TCCGGAATTA TTGGGCGTAA AGCGCTCGCA
 541 GGCGGGTTCT TTAAGTCTGA TGTGAAATCT TGCGGCTCAA CCGCAAA

Sample 2: 1 CAAGCGTTGT CCGGAATTAT TGGGCGTAAA GCGCTCGCAG GCGGTCTTT AAGTCTGATG
 61 TGAAATCTCG CGGCTCAACC CGAACGGTC ATTGGAAACT GGAGGACTTG AGTACAGAAG
 121 AGGAGAGTGG AATTCCACGT GTAGCGGTGA AATGCGTAGA GATGTGGAGG AACACCAGTG
 181 GCGAAGGCGA CTCTCTGGTC TGTAACTGAC GCTGAGGAGC GAAAGCGTGG GGAGCGAACA
 241 GGATTAGATA CCCTGGTAGT CCACGCCGTA AACGATGAGT GCTAGGTGTT AGGGGGTTTC
 301 CGCCCCTTAG TGCTGAAGTT AACGCATTA GCACCTCCGCC TGGGGAGTAC GGCGCAGG
 361 CTGAAACTCA AAAGAATTGA CGGGGGCCCG CACAAGCGGT GGAGCATGTG GTTTAATTG
 421 AAGCAACGCG AAGAACCTTA CCAGGTCTTG ACATCCTGTG CTATTCTTAG AGATAGGAAG
 481 TTCCCTTCGG GGACAGAGTG ACAGGTGGTG CATGGTTGTC GTCAGCTCGT GTCGTGAGAT
 541 GTTGGGTTAA GTCCCGAAC GAGCGAACCTTAA GTTGATCTTA GTTGCCAGCA TTTAGTTGGG
 601 CACTCTAAGG TGACTGCCGG TGACAAACCG GAGGAAGGTG GGGATGACGT CAAATCATCA

661 TGCCCCTTAT GACCTGGGCT ACACACGTGC TACAATGGAT GGAACAAAGG GAAGCAAAAC
721 CGCGAGGTCA AGCAAATCCC ATAAAACCAT TCTCAGTCG GATTGCAGGC TGCAACTCGC
781 CTGCATGAAG CCGGAATCGC TAGTAATCGC GGATCAGCAT GCCGCGGTGA ATACGTTCCC
841 GGGCCTGTAA CACACCGCCC GTCACACCCAC GAGAGTT

Figure (4): FASTA Sequence of the new 2 bacterial strains.

A official website of the United States government [Here's how you know](#)

NIH National Library of Medicine
National Center for Biotechnology Information

Nucleotide Nucleotide Advanced

GenBank ▾ Send to: ▾

Figure (5): Registration documents of the new bacterial strains in NCBI.



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