

ARTICLE / INVESTIGACIÓN

Isolation and molecular identification of *Exiguobacterium profundum* from drain water and study of some physiological properties

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DOI. 10.21931/RB/2022.07.02.16

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Abstract: Some microorganisms' stress tolerance and adaptability through the ability to live in new environments previously considered hostile to dense microbial growth. The aim of the study: In this work, the isolation, molecular identification, and analysis of some physiological properties of *Exiguobacterium profundum* isolated from washing machine drain water were carried out. Fifteen samples were collected from different washing machines' drain water, and 42 isolates were characterized and identified. Based on the morphological assay and 16S rRNA gene sequencing, one isolate was designated as *Exiguobacterium profundum* Kh-Am2 and was deposited in the Gene bank database under the accession number MW447498.1. The physiological properties assay showed that *E. profundum* could not decompose red blood cells while proving their ability to decompose crude and coconut oil, producing lipopeptide biosurfactants. It has also demonstrated its ability to form biofilm using the tube method, which helps it to live in harsh environments such as the environment of washing machines. The results showed that *E. profundum* is halotolerant bacteria with its ability to grow at a concentration of (75) g/l NaCl and the best growth was at(25) g/l NaCl concentration. The results also showed the effect of different concentrations of NaCl on *E. profundum*, where the best movement rate was at a concentration of (25)g/l NaCl, while the movement was completely inhibited at a concentration of (75)g/l NaCl. We have concluded that *E. profundum* possesses traits that enable it to live in harsh environments.

Key words: *Exiguobacterium profundum*, drain water, biosurfactant.

Introduction

Exiguobacterium is a bacterial genus with a wide range of species obtained from various habitats. *Exiguobacterium* first was recognized as a genus about three decades ago, and there are presently Seventeen species in this genus. *Exiguobacterium* is a halotolerant bacteria that has piqued the interest of many researchers as a crucial agent in salinity stress reduction, capable of thriving in a wide range of temperatures and pH¹.

Exiguobacterium isolates have been employed in biotechnology and industrial applications such as enzyme synthesis, bioremediation, and the breakdown of toxic materials released into the environment. Other isolates can promote plant development, and they are currently being investigated to enhance agricultural production. In the genomic sequences of diverse strains of this species, many genes encoding products crucial to agriculture and the environment have been found. In the genomic sequences of various strains of this species, many genes encoding products important to agriculture and the environment have been found. Many strains also feature stress-response genes, allowing them to colonize and thrive in a wide range of ecological settings^{1,2}. *Exiguobacterium* species such as "*E. sibiricum* , *E. alakliphilum* , *E. undae*, *E. oxidotolerans*, *E. mexicanum* , *E. antarcticum*, *E. profundum*, and *E. indicum*" have been discovered in a variety of environments³ .

E. profundum is a species of *Exiguobacterium*, Gram-positive, nonsporulating rods that can be found mono, pairs or

in short motile chains with peritrichous flagella. Colonies are spherical (1–2 mm) and creamy or orange in color under anaerobic or aerobic conditions. Chemo-organ trophic and facultatively anaerobic. Positive for catalase and negative for oxidase. It is slightly thermophilic (growing within 12 - 49 °C, optimum at 45 °C) and halotolerant (growth in 11 percent NaCl, optimal 0–2 percent NaCl). Its pH range for growth is 5.5–9.5. (Optimum pH 7.0). Dextrose, lactose, maltose, mannose, and sucrose were used to test carbohydrate fermentation. The major organic acid produced by carbohydrate fermentation is L-Lactate, with traces of formate, ethanol, and acetate also present. Mueller Hinton broth, "Soybean Casein Digest broth, King's broth, Mac Conkey broth, and Nutrient broth media" were used to assess the maximum growth and pigment production properties^{4,5}.

According to several studies, *Exiguobacterium profundum* can be used in bioremediation and toxic degradation by producing lipopeptide biosurfactants, which have stable chemical characteristics, are renewable, environmentally beneficial, and have low toxicity^{2,6,7}.

Currently used in industry, cosmetics, and pharmaceutical emulsions, foaming agents, wetting agents, emollients, and have antibacterial activity against gram-negative bacteria^{2,8,9}.

This research aimed to isolate and molecular identify *Exiguobacterium profundum* and study some physiological properties such as biosurfactant production, biofilm production, and salinity on growth and motility.

Citation: G.O. Al-Ani A, Mohammed Younis K, M.Y. Al-Tae S. Isolation and molecular identification of *Exiguobacterium profundum* from drain water and study of some physiological properties. *Revis Bionatura* 2022;7(2) 16. <http://dx.doi.org/10.21931/RB/2022.07.02.16>

Received: 30 November 2021 / **Accepted:** 25 January 2022 / **Published:** 15 May 2022

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Materials and methods

Sample Collection and Isolation

Fifteen samples were collected from washing machines, drained water in sterile containers and brought to the laboratory. Following, 0.1 mL from each sample was cultured on Trypto Soy Agar (TSA) by using sterile swab sticks (BIONOVO, Poland). The plates were incubated at 37°C for 24-48 h. After the incubation, isolates were purified to obtain single colonies, then underwent to identification by using Gram-stain, colonies appearance and molecularly identified by using the 16S rRNA gene and the following assays¹⁰.

Amplification of 16S rRNA gene and Phylogenetic Tree

DNA extraction

The isolate was grown at 37 °C for 24 h on a rotary shaker (250 rpm) in a falcon tube containing 20 mL of Tryptic Soy Broth (TSB). The method for extracting DNA was subsequently followed using the G-spin DNA extraction kit (intron biotechnology, Korea). The 16S rRNA genes were amplified by polymerase chain reaction (PCR) from genomic DNA of selected isolates according to the Maxime PCR (PreMix kit) methodology. For PCR reaction, 1.5µl of DNA template, 1µl of 10 picomols/µl of each conserved primers 1250R (5'-GGTTACCTTGTTACGACTT-3') and 1250 F (5'-AGAGTTTGATCCTGGCTCAG-3') from (Integrated DNA Technologies company, Canada), 5µl of Taq PCR PreMix and 16.5 µl ddH₂O for a total volume of 25µl. PCR with the optimal cycle setting for the detecting 16S rRNA gene was carried out using a Bio-Rad MyCycler thermal cycler (Bio-Rad, USA) with an initial denaturation step at 95°C for 5 min, Denaturation step at 95°C for 45 sec, 35 cycles of 95°C for 45 sec, 58°C for 45 min, 72°C for 45 sec, followed by a final extension step of 72°C for 7 min. Amplification products were analyzed by electrophoresis in 1% (w/v) agarose gel.

Purification of PCR Products

According to the manufacturer's instructions, the PCR products were purified using the QIAquick PCR purification kit (QIAGEN). In brief, one volume of PCR product mixed with five volumes of Binding Buffer was mixed thoroughly and, after that, placed in a QIAquick spin column, followed by spinning for 60 seconds to bind the DNA, flow-through was removed, the column was then placed back into the collecting tube. DNA was centrifuged for 60 seconds after being cleaned with 0.75 ml of washing buffer. The flow was removed, and the tubes were centrifuged for another minute. After that, the column was put in a fresh 1.5 ml centrifuge tube. Fifty µl Buffer of Elution was added to the middle of the QIAquick membrane for DNA elution, and the tubes were centrifuged for 1 minute. Macro gene/Korea Sequencing Service sequenced the purified PCR products. (Following Kit instructions).

Phylogenetic tree

The DNA sequence was collected and matched to sequences in the gene bank databases. Homolog surveys were carried out using internet bioinformatics tools (www.ncbi.nlm.nih.gov/BLAST). Sequences had a lot of similarities (100%) with species *E. profundum*. In FASTA format, all isolates were retrieved and downloaded. A maximum-likeli-

hood Multiple Sequence Comparative by Log Expectation (MUSCLE) software created phylogenetic trees with the 16S gene of Kh-Am2 strain using program MEGA version 7.0 and matched sequences. The confidence value of individual branches was calculated using 1000 bootstrap copies of the original sequences. The complete 16S rRNA gene sequences were deposited in the Gene Bank database.

Biosurfactant screening

Blood agar hemolysis assay

Sterile fresh blood in volume 1.5 mL was added to a 100 mL Erlenmeyer flask containing a sterile blood base medium. The selected isolate was streaked onto blood agar and cultured for 48-72 hours at 37°C⁹.

Oil spreading assay

The method of (9) was used in the screening of biosurfactants production for Kh-Am2 isolate. Using a petri dish, 30 mL of distilled water was poured into it, 1 ml of coconut oil and crude oil were separately tubed and dropped into the center; in the center of the oil layer, 20 mL of the Kh-Am2 culture was placed.

Spread Plate assay

Following the method of Spread Plate assay with modified¹⁰, the assay was done to conduct the ability of the selected isolate to consume crude oil and coconut oil. The plates containing Bushnell Hass medium (BHM) as a mineral salt medium comprised of (K₂HPO₄ 1gm, MgSo₄.7H₂O 0.2 gm, FeCl₃ 0.05 gm, CaCl₂ 0.02 gm, NH₄NO₃ 1gm, , Agar 15 gm) in 1L of distal water were prepared with added 1% of crude oil and coconut oil separately. Each plate was inoculated by 0.1 ml 1.5×10⁸ cells ml⁻¹ of selected isolate suspension. Then plates were incubated for seven days at 37°C. The growth of colonies after the incubation time is taken as a sign of their ability to use crude and coconut oil as a source of energy and carbon.

Biofilm formation detection using the tube method

The chosen isolate was inoculated in a tube containing ten trypticase soy broth and 1% glucose and cultured for 24 hours at 37°C. After incubation, tubes were poured and washed with phosphate buffer saline (pH 7.3) before drying. The tube was then colored with crystal violet (0.1 percent). Any residual discoloration was removed with deionized water and then dried inside out. The findings of the control were used to evaluate the tube method. It was considered a positive sign of biofilm development when a visible film lined the tube's wall and bottom^{11,12}.

Salinity tolerance

The Kh-Am 2 isolate was cultivated on YP culture media with NaCl concentrations of (0), (25), (50) and (75) g/l. 75 hours at 25°C incubation with orbital agitation at 120 rpm. OD₆₀₀ was measured in a UV-visible spectrophotometer UV 1800 to track growth.

Salinity's Influence on Motility

The selected isolate was cultivated on YP agar (0.3% agar / Difco). A drop over the agar in the Petri plate's middle inoculated. The motility ratio were evaluated after 72 hours of incubation at 25 °C in (0), (25), (50) and (75) g / l NaCl¹³.

Results and discussion

The streaking results on cultured medium plates revealed that the drain water samples contain various microbial communities with different gram-positive and gram-negative bacteria. A total of 42 bacterial isolates were isolated. The target Gram-positive rods bacteria of *Exiguobacterium* was the most numerous of other isolates, and this agrees with what was indicated by both^{4,14} and was chosen for further studies.

16S rRNA gene sequence and phylogenetic analysis

The isolate designated as the Kh-Am2 and their 16SrRNA gene sequences was recorded in the NCBI Gene bank databases under the accession number MW447498.1. Once the 16S rRNA gene of the Kh-Am2 isolate was amplified and analyzed using a 1 percent agarose gel-electrophoretic method, the PCR result was 1500 bp, as shown in Gene data (<http://www.ncbi.nlm.nih.gov>) a search tool was used to perform BLAST analysis on partial 16S rRNA sequences. The Kh-Am2 isolate exhibited 99-to-100% matched with the NCBI Gen database's sequences. The isolate's Neighbour Joining tree as constructed in MEGA version 7 (Figure 1), identified that a neighbor-joining tree based on similar 16S rRNA gene sequences for the *E. profundum* Kh-Am2 is the most closely related to *Exiguobacterium* sp. 8A. They are both clustered with *E. profundum* strain fsznc-03 and *E. profundum* strain GN31a.

Blood agar hemolysis assay

The results of the hemolysis test on the blood agar me-

dium showed a negative result indicating that no clear areas were formed around the inoculation line, which is consistent with what was indicated by both⁹ as it does not produce hemolysin that breaks down the red blood cells.

Oil spreading assay

The results showed the ability of Kh-Am2 isolate to produce biosurfactant in terms of clear zones and oil displacement zones for both types of crude oil and coconut oil, as shown in (figure 2). The reason is due to the ability of Kh-Am2 to produce biosurfactant; in addition, *Exiguobacterium* strains produce alkali esterase enzyme

belonging to Lipolytic enzymes²², and this is consistent with what was referred to by (9), who stated that The biosurfactant produced by *E. profundum* could be classified into the lipopeptide group, It also gave the results of the oil spreading assay presented by Ahmed's observation for *Bacillus cereus* ND 1, as it showed its ability to decompose crude oil¹⁵, Biosurfactants work as emulsifiers by lowering surface tension whereas, biosurfactants are made up of polar and nonpolar parts¹⁶.

Spread Plate assay

Kh-Am2 isolate showed its ability for growing on BHM containing crude oil and coconut oil separately, which indicates its ability to consume both crude oil and coconut oil, as shown in (figure 3), due to its ability to use crude oil and coconut oil as a source of energy and carbon. The researcher¹⁷ pointed out the role of *Exiguobacterium* sp. AO-11 in removing crude oil pollutants from the microcosms of sediments, and The researcher Chen referred to the role of *Exiaubacterium* sp in the decomposition of crude oil¹⁸.

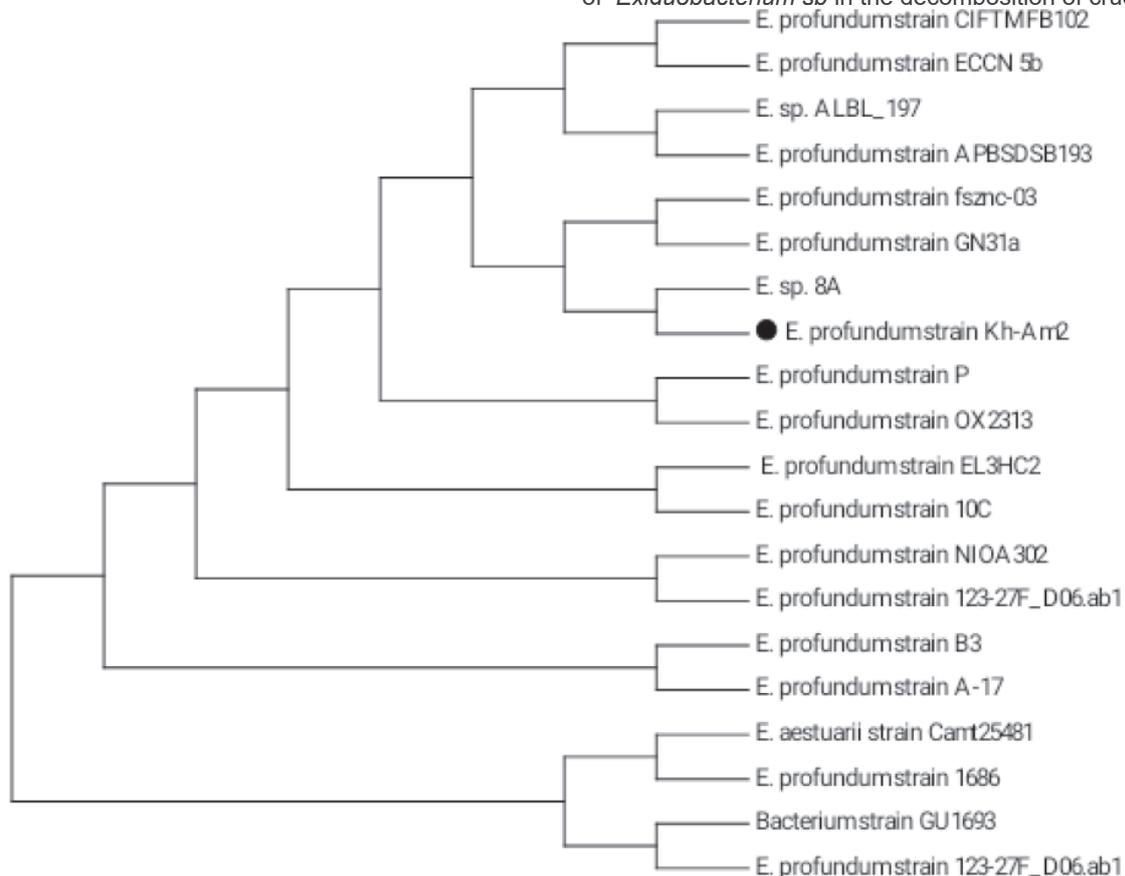


Figure 1. Phylogenetic tree (1000 bootstrap) constructed by MEGA7 tool from 19 merged nucleotide sequences of 16S rRNA gene homologous to that of *Exiguobacterium profundum* strain Kh-Am2 (indicated by black cycle).

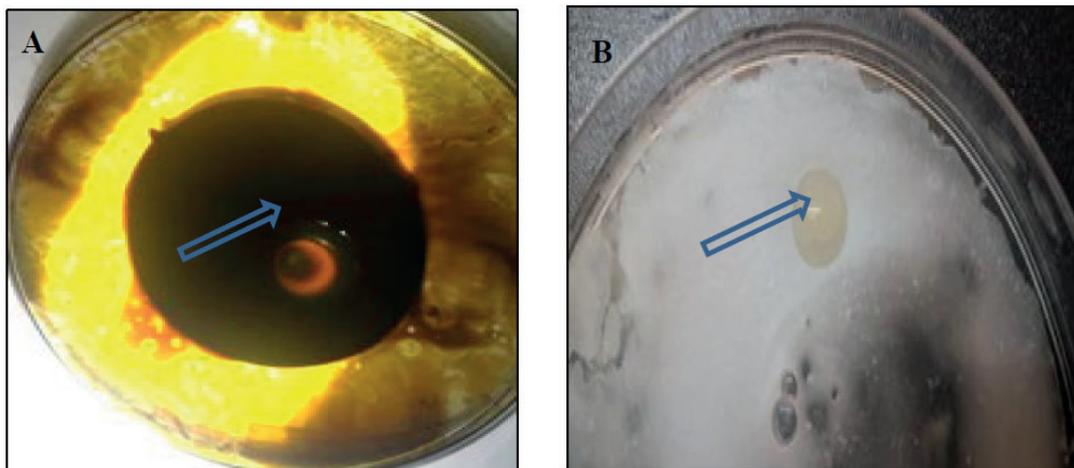


Figure 2. The clear zone begins to form in the oil spreading assay:- (A) crude oil (B) coconut oil.

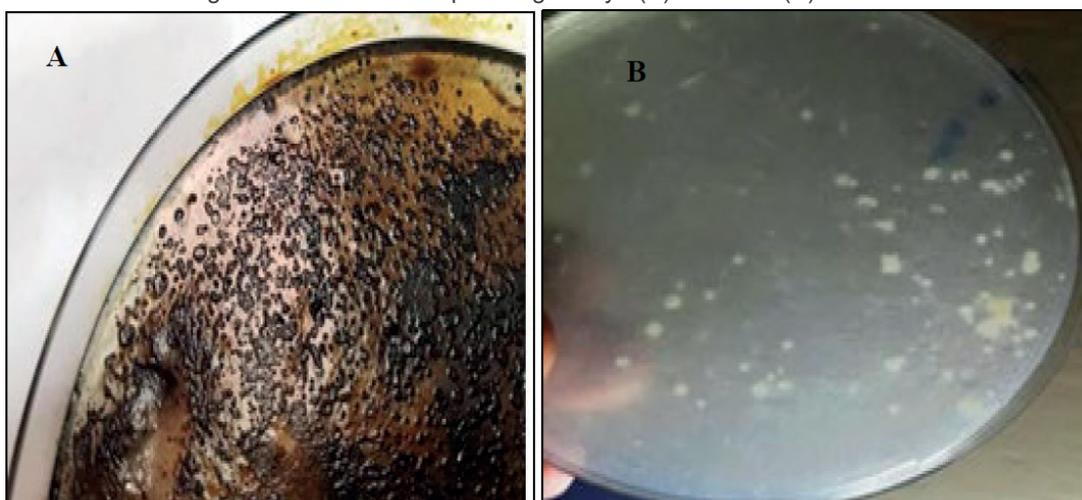


Figure 3. The ability of Kh-Am2 isolate to consume crude and coconut oil on BHM :- A(BHM with 1% crude oil) B (BHM with 1% coconut oil).

This could be a unique trait that helps to the survival of these populations and reflects the ability of these creatures to thrive in such harsh conditions in the washing machines, as it can depend on its growth on oils sticking to clothes when it comes out with drain water.

Biofilm formation detection using the tube method

The results showed the ability of Kh-Am2 to adhere and form a biofilm by using the tube method in terms of the deposition of the crystal violet on the inner side of the test tube, as shown in (figure 4). Biofilms are a community of bacteria that produce extracellular substances such as proteins, polysaccharides, DNA, RNA, and water. The essential component of a biofilm is the nutrient flow control matrix¹⁹. The ability of Kh-Am2 isolate to generate biofilms was demonstrated using the biofilm tube approach. Where the researcher¹⁷ was referring to *Exiguobacterium* sp. ability to create biofilm. Biofilm production is one of the most important mechanisms that the Kh-Am2 isolate has for surviving in harsh settings.

Salinity tolerance

The results showed the ability of Kh-Am2 isolate to grow in high concentrations of salt. The best growth was found in (25) g/l of NaCl, where the absorbance reached 0.8 at OD₆₀₀ nm. The concentrations (0), (50), (75) g/l of NaCl were 0.4, 0.3, 0.3 at OD₆₀₀, respectively, as shown in (figure 5), where it gave a similar reading for both concen-

trations (50) and (75) g/l of NaCl, this result makes the Kh-Am2 isolate is classed as halotolerant bacteria, While the researcher¹³ indicated that *Exiguobacterium* sp. SH31 were unable to grow at a concentration of (75) g/l NaCl percent, but they were able to grow at a salt concentration of (50) g/l NaCl percent¹⁹ also noted that the growth of *E. profundum* PHM11 stopped at the concentration of 2500 mM. There is little published research on how *Exiguobacterium* tolerates salinity by fine-tuning its gene expression patterns and modifying its inherent physiology. Living in extreme environments causes their genes to adapt to harsh environmental conditions, and thus the enzymes and proteins encoded by the genes can withstand extreme conditions.

Salinity's Influence on Motility

The results showed that Kh-Am2 isolate moved (swimming movement) on YP agar at (0), (25) and (50) g/l NaCl, where the highest percentage of movement was observed at a salt concentration of (25) g/l NaCl, which represented the best growth for the isolate as observed in (Figure 6). At the same time, the motility was wholly inhibited at (75) g/l NaCl as shown in (figure 6).

Some bacteria respond to osmotic pressure, as in *Pseudomonas putida*²¹. Although *Exiguobacterium* bacteria are described as motile²², it suggests that specific salt concentrations make the cell less active or could be gover-

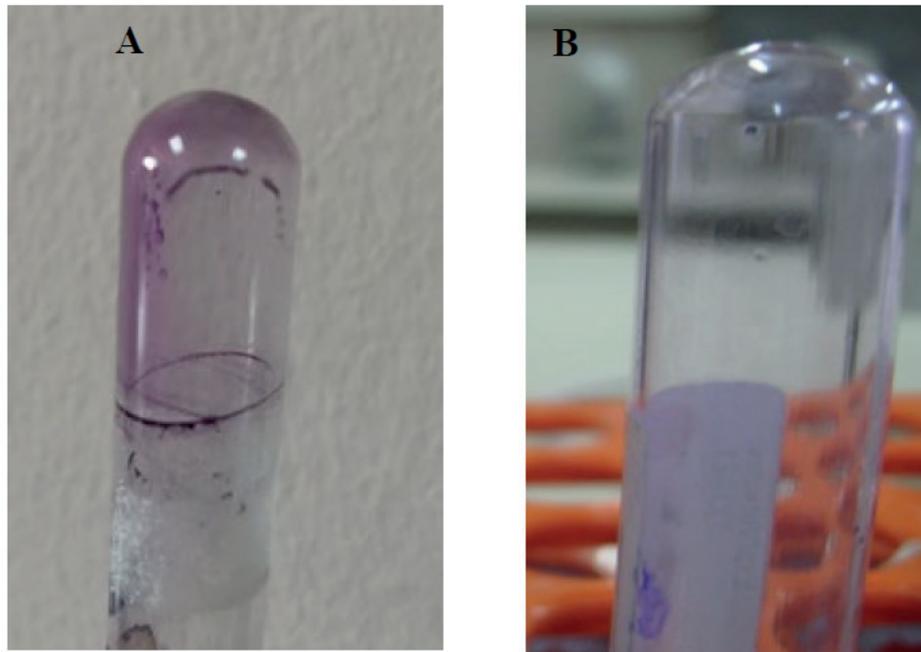


Figure 4. Biofilm formation by Kh-Am2 on the inner sides of the test tube. A:- Positive result; B:-Control.

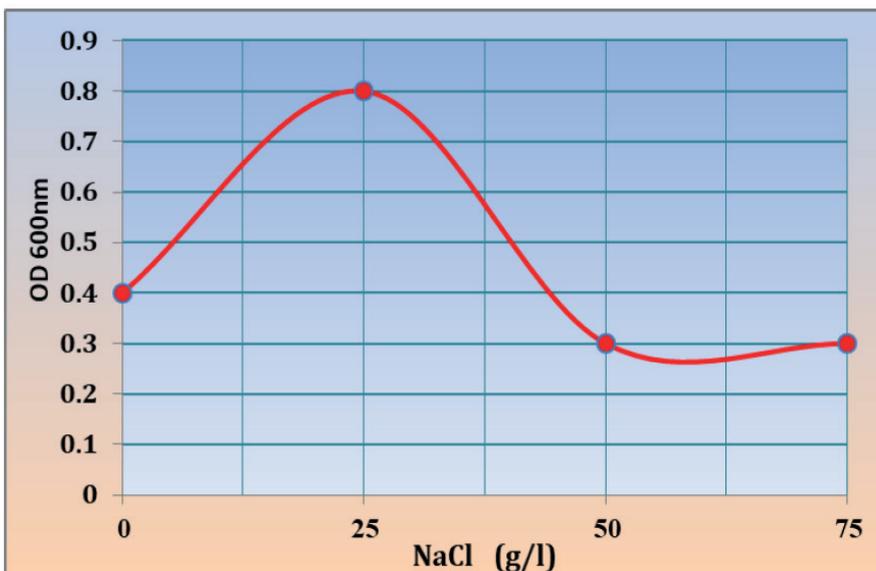


Figure 5. Growth of Kh-Am2 in 0, 25, 50 and 75 g/l NaCl.

ned by environmental factors somehow²³. Flagella synthesis requires much energy, and bacteria can block specific processes in reaction to stress. Motility was reduced, and biofilm development was inhibited¹³. The results of salinity on motility showed that the highest motility (Swimming motility) was obtained at a concentration of (25) g/l NaCl. In contrast, motility was inhibited at a concentration of (75) g/l NaCl, and this agrees with the scientist (13) in obtaining the highest percentage of motility at a concentration of (25) g/l NaCl for *Exiguobacterium sp.* SH31 while the movement was inhibited at a (50) g/l NaCl concentration.

Conclusions

Under extreme conditions, salinity is a significant element in determining the spread of life. Our study suggests that *E. profundum* Kh-Am2 possesses a stressing regulation system that controls gene expression and supports the organism's survival and growth in salt environments. Our

data revealed the potential of the *E. profundum* Kh-Am2 isolate to grow at a high salt concentration of 75 g/L and the ability to develop a biofilm. This was related to maintaining the integrity of bio-molecules in the physiological process and preserving cell architecture and function. *E. profundum* Kh-Am2 can degrade crude and coconut oil in an oil spreading assay, indicating that the bacteria can be used in bioremediation and toxic degradation.

More analyses are needed to comprehend further the gene regulation of salinity pathway of the *E. profundum* Kh-Am2 isolated from waste water of a washing machine.

Acknowledgment

The researchers would like to express their gratitude to the University of Mosul for supporting this study. The tools were also provided by the Biology department and the Iraqi Ministry of Higher Education and Scientific Research.

Conflict of interest

The authors declare that they have no conflict of interest.

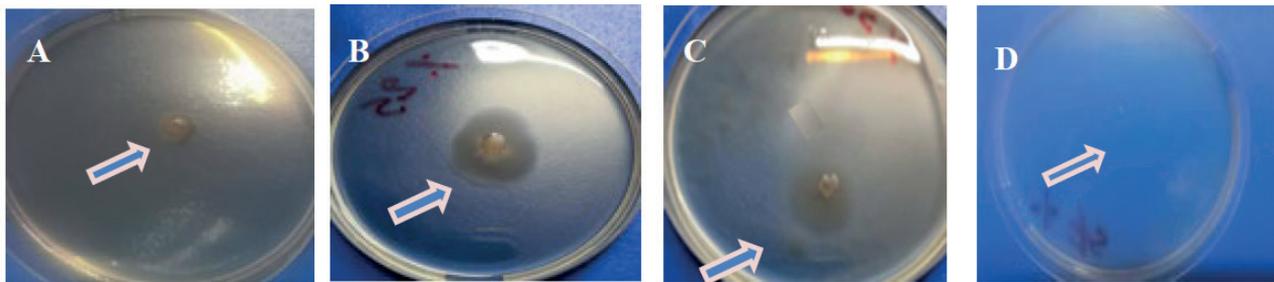


Figure 6. Effect of salinity in the motility of Kh-Am2 with different concentrations of NaCl after incubation 72 h at 25°C. A .Concentration 0 g/l of NaCl, B.Concentration 25 g/l of NaCl, C .Concentration 50 g/l of NaCl, D.Concentration 75 g/l of NaCl.

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Funding

This research did not receive any specific support from public or commercial funding agencies.

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