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Title: Isolation and characterization of Zinc Solubilizing Bacteria in Erbil Governorate soils

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ABSTRACT

Zinc (Zn) is an essential micronutrient to support many physiological and biochemical functions in plants, such as enzyme activation, protein synthesis, and growth regulation. But bioavailability in calcareous soils is limited, like those found in many production areas including Erbil Governorate, Iraq. Cause by high soil pH, and zinc can become tied up in indissoluble forms resulting in reduced availability to plants. To solve this problem the current study intended to isolate, characterize, and molecularly identify zinc-solubilizing bacteria (ZSB) from agricultural soils in the Erbil area. Soil samples were collected from the rhizosphere zones of different cultivated plants because the levels of microbial activity are usually greater in these locations. The bacterial isolates were screened for their Zn-solubilizing ability employing the Pikovskaya's agar medium, with zinc oxide (ZnO) as a poorly soluble form of zinc. Seventy-one (71) bacterial isolates were isolated and analyzed. Three of these isolates (*Bacillus subtilis, Delftia tsuruhatensis*, and *Pseudoxanthomonas mexicana*) had much better Zn solubilizing ability, each with a clear halo and were determined to have solubilization indices (SI) > 2.5. The bacterial isolates were identified with morphological, biochemically, and molecular analysis using 16S rRNA gene . Quantitative analysis of the strains' growth in liquid culture presented via Atomic Absorption Spectroscopy (AAS) showed solubilising large amounts of zinc. Overall, it is conceivable that the identified ZSB isolates might provide excellent tools as bio-fertilizers for increasing zinc availability in soils with zinc deficiency, leading to better plant growth and improved soil health. The use of these indigenous strains may represent an environmentally friendly and sustainable strategy for maximizing agricultural productivity in the area.

***Keywords*:** ZSB, Pikovskaya's agar, 16S rRNA , *Bacillus subtilus ,psudoxanthomonas Mexicans and Delftia tsuruhatnsis*.

INTRODUCTION

One of the most important roles performed by bacteria in nature is the biogeochemical cycling of zinc, which facilitates the decomposition of organic molecules essential to plant growth. As a result of these biogeochemical processes, microorganisms weather zinc compounds in rocks and minerals, transforming insoluble forms of zinc into soluble and bioavailable zinc [1] and [2]. Zinc-solubilizing bacteria (ZSB) convert infeasible zinc to plant usable forms of zinc (Zn^{2+}) using a host of redox processes, chelation, and acidification [3]. ZSB are shown to create an environment that either harness or produce organic acids (facilitating availability) that can yield soluble Zn in the soil [4] and [5]. This occurs by producing gluconic, citric and acetic acids that solubilize zinc in insoluble zinc compounds and thereby decreases soil pH [4] and [5]. In addition, ZSB generates siderophores that chelate zinc, thereby enhancing the accessible surface area for the plant roots. They also release phytohormones like auxins, which increase zinc absorption and encourage root growth [6] and [7] .It is thought that a large number of microorganisms contribute to the solubilization of insoluble zinc. For the advantage of interacting ecosystems in a purely microscale setting, some members of soil microbial communities determine the availability of nutrients, including zinc, in soil aggregates. Numerous vital micronutrients, which are only found in trace amounts, are lacking in Iranian soils. Zinc is one of the essential elements with a growth deficit [8] and [9] .Moreover, zinc is necessary for numerous enzymes involved in energy transmission, protein synthesis, and nitrogen metabolism [10] .Rhizobium, Pseudomonas, and Bacillus are common ZSB genera [11] .The development, activity, diversity, and spread of microorganisms are all necessary for

the biogeochemical cycling of zinc [12] and [13]. The goal of the current study was to use molecular analysis to isolate and characterize zinc-solubilizing bacteria from Erbil soils and to assess the isolate bacterial strain's zinc solubilizing efficiency (ZSE). Finding and analyzing bacteria from Erbil soils that can transform unavailable zinc into a form that plants can absorb is the goal of the study. This is crucial for plant growth and soil health, particularly in areas with zinc deficiencies.

Material and Methods

2.1. Collection of soil samples

Soil samples obtained from different topographical areas in Erbil governorate/Kurdistan region-Iraq in September 2024 from 15 cm of surface soil included (Khabat, Kawrugosk, Grdarasha, Qushtapa, Halyawa, Shamamar, Gure, Malaqara, Mxmur, Dybaga, Xalifan, Haji omeran, Kore, Salahadin, Shaqlawa, Choman, Dyana, Harir, Galala and Rawandz). The soil samples were subsequently transferred to Microbiology laboratory, Department of Plant Protection, Salahaddin University's, College of Agricultural Engineering Sciences.

2.2. Isolations and Identifications of Isolate Bacteria

Using a series of dilutions, soil samples were cultivated on a plate of Biotite containing Pikovskaya (The following are measured in grams per liter: 1.000 yeast extract, 10.000 mannitol, 0.500 dipotassium phosphate, 0.200 magnesium sulphate, 0.100 sodium chloride, 15,000 calcium carbonate, and 1.000 agar) in order to isolate the bacteria that dissolve zinc and adding by unsoluble zinc compound (ZnO)) agar medium [14], correspondingly, in accordance with Anderson and Pascual's 2000 protocols. Bergey's Manual for Determinative Bacteriology states that, microscopical, morphological, biochemical, and physiological tests were performed following a 75-hour incubation period at 28°C. included (gelatin liquefaction, nitrate reduction, aerobic test, oxidase and catalase test, colony morphology, color, motility, spore production, cell shape, gram stain,[15] and [16] Each strain's molecular identity was determined using the 16S rRNA partial sequencing. Universal bacterial primers were used to extract and amplify the DNA: The 16srRNA primers (5' to 3') include the following sequence: F: 5'CGTTGACTGCCGGGGACAAAC'3. PCR buffer, deoxynucleotide triphosphates, gel loading dyes, Taq DNA polymerase, and Novel Green dye make up this enhanced ready-to-use 2× PCR mixture. It generates a fluorescence dye that can be seen right away following DNA electrophoresis when exposed to ultraviolet light or a blue-light transilluminator. Everything that is PCR competent—aside from the primer and DNA template—is included in the Master Mix. The PCR condition and amplicon size for 16S rRNA gene understudy as follow for identifying the organisms, DNA amplification for the 16S rRNA gene was performed in the heat cycler for five minutes at 95˚C to guarantee that the DNA templates are completely denaturated After that, the PCR was carried out using the following protocol: denaturation for 40 seconds at 95°C, annealing for 45 seconds at 59°C, and extension for 45 seconds at 72°C. These parts were repeated forty times, with a final extension of ten minutes at 72°C. PCR tubes were then kept at -20°C until they could be examined further. obtained sequence were aligned with reference RNA sequence from Macrogen (Korea).

2.3. Preservations of Bacterial Isolates

For additional research, the recovered bacteria's pure colonies were kept on agar slants at 4 °C and at -75 °C with 25% glycerol [17].

2.4.Statistical Analysis

Using SPSS 16.0, Duncan's H.S.D. multiple range tests were used in every instance to compare treatment means [18].

2.5. Zinc's effectiveness Dissolving Zinc-containing bacteria Soil Solubilization:

Using the plate screening method, the zinc solubilizing effectiveness of each isolate was evaluated. Spot inoculation was performed at the center of the PVK plate, and all suspended colonies were evaluated for zinc solubilization effectiveness on PVK ager medium supplemented with zinc oxide insoluble substance. after 28±2˚C incubation. After 24 hours and up to 7 days, the clear halo diameter was successfully measured. Efficiency of zinc solubilization Each isolate's levels of *Bacillus subtilis*, *Delftia tsuruhatnsis*, and *Psudoxanthomonas Mexicana* were assessed using the following formula [19]: Solubilization Efficiency is S.E.

S.E= halo zone Diameter/ colony diameter × 100

The most efficient isolates were selected.

2.6. Molecular identifications of zinc solubilizing bacteria

Extraction of genomic DNA from bacterial culture cells

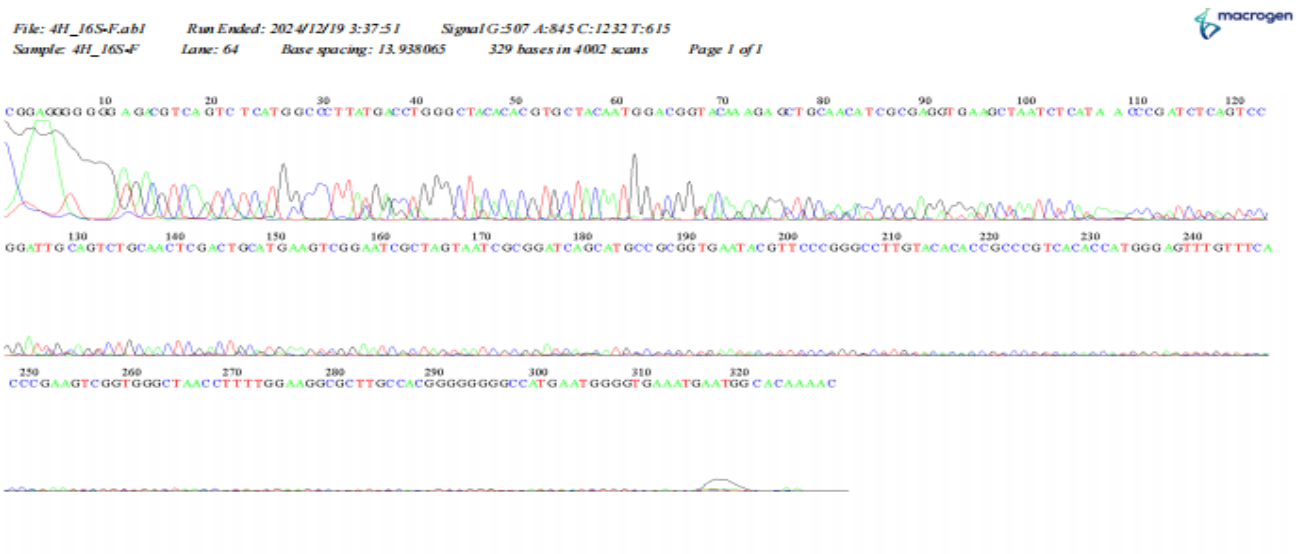
Genomic DNA was extracted from pure cultures through the GeneAll® ExgeneTM for Clinic Cell SV mini kit

(Songpa-gu, Seoul, KOREA). Harvested cultural cell should be used freshly. During lysis incubation, shaking or vortexing can significantly increase lysis efficiency and shorten the time needed for full lysis.
The PCR technique was used to amplify the genes of bacteria. The primer milting temperatures (Tm) were followed for setting up the PCR procedure in 25 uL reactions. The PCR condition and amplicon size for 16S rRNA gene understudy as follow for identifying the organisms, amplified DNA for the 16S rRNA gene was performed in the heat cycler for five minutes at 95˚C to guarantee that the DNA templates are completely denaturated. The following program was then used to continue the PCR: 40 seconds of denaturation at 95°C, 45 seconds of annealing at 59°C, and an extension at 72°C at 45 sec. These parts were repeated forty times, with a final extension of ten minutes at 72°C. PCR tubes were then kept at -20°C for additional examination, in turn. The amplified genes' sizes were verified using A 1.5 percent agarose gel and UV detection using ethidium bromide.

Sequencing of bacterias are :

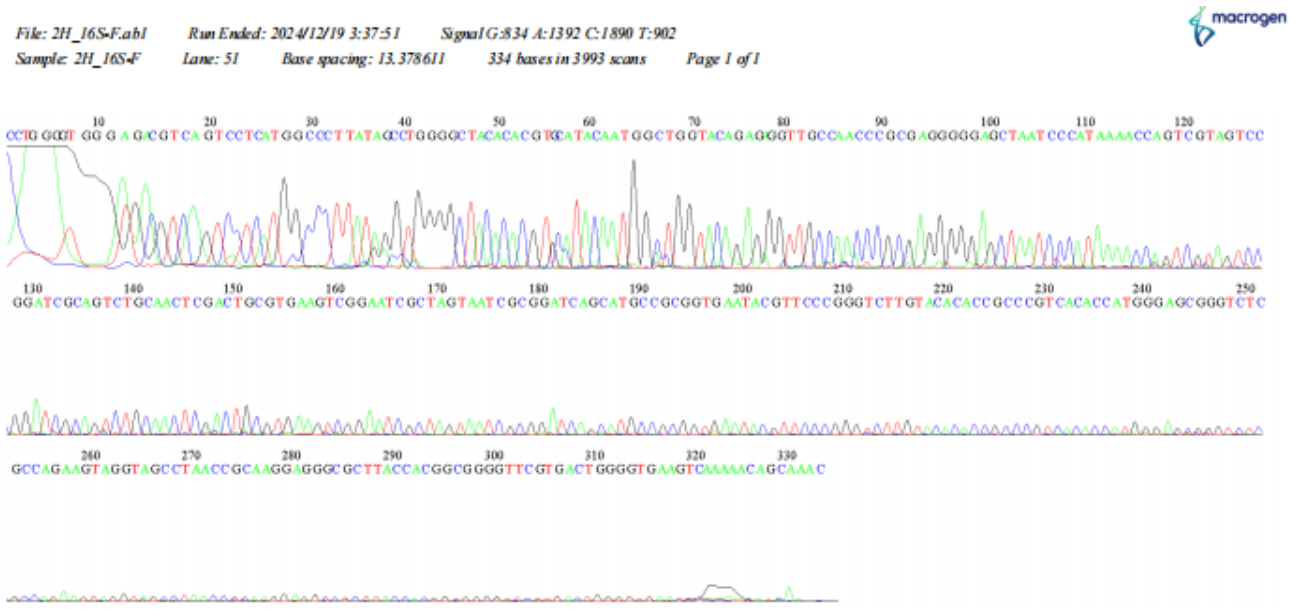
1- (*Bacillus subtilus)*

CGGAGGGGGGGAGACGTCAGTCTCATGGCCCTTATGACCTGGGCTACACA
CGTGCTACAATGGACGGTACAAAGAGCTGCAACATCGCGAGGTGAAGCTA
ATCTCATAACCCGATCTCAGTCCGGATTGCAGTCTGCAACTCGACTGCAT
GAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGT
TCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTTTCACC
CGAAGTCGGTGGGCTAACCTTTTGGAAGGCGCTTGCCACGGGGGGGGCCA
TGAATGGGGTGAAATGAATGGCACAAAAC



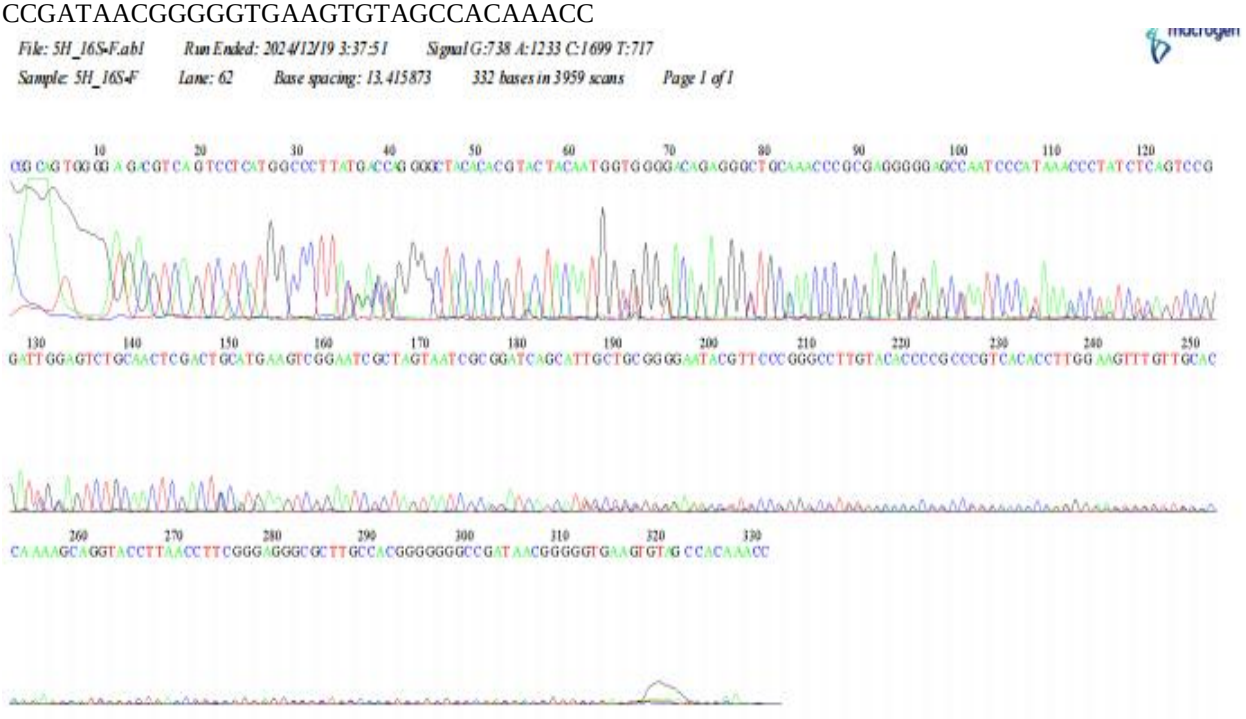
2- (*Delftia tsuruhatensis)*

CCTGGGGTGGGAGACGTCAGTCCTCATGGCCCTTATAGCCTGGGGCTACA
CACGTGCATACAATGGCTGGTACAGAGAGGTTGCCAACCCGCGAGGGGGA
GCTAATCCCATAAAACCAGTCGTAGTCCGGATCGCAGTCTGCAACTCGAC
TGCGTGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAA
TACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGGGAGCGGGTC
TCGCCAGAAGTAGGTAGCCTAACCGCAAGGAGGGCGCTTACCACGGCGGG
GTTCGTGACTGGGGTGAAGTCAAAAACAGCAAAC



3 -(*psudoxanthomonas Mexicans)*

CCGCAGTGGGGAGACGTCAGTCCTCATGGCCCTTATGACCAGGGGCTACA
CACGTACTACAATGGTGGGGACAGAGGGCTGCAAACCCGCGAGGGGGAGC
CAATCCCATAAACCCTATCTCAGTCCGGATTGGAGTCTGCAACTCGACTG
CATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATTGCTGCGGGGAAT
ACGTTCCCGGGCCTTGTACACCCCGCCCGTCACACCTTGGAAGTTTGTTG
CACCAAAAGCAGGTACCTTAACCTTCGGGAGGGCGCTTGCCACGGGGGGG
CCGATAACGGGGGTGAAGTGTAGCCACAAACC



3. Results and Discussion

3.1. Identification and Isolation of Bacteria That Solubilize Zinc

Seventy-one isolates of bacteria that dissolve zinc have been collected and examined from different rhizosphere soil locations in the Iraqi Kurdistan area and Erbil governorate(Ch1,Ch2,Ch3,Ch4,Ch5 ,Dib6,Dib7,Dib8,Sha9,Sha10,Sha11,Haj12,Haj13,Haj14 ,Raw15,Raw16,Raw17,Raw18,Raw19,Qus20,Qus21,Qus22,Grd23,Grd24,Gal25,Gal26,Gal27,Gal28,Xab29,Xab30, Xab31,Kor32,Kor33,Kor34 ,Kha35,Kha36,Kha37,Aly38,Aly39,Aly40,Aly41,Har42,Har43,Har44,Dya45,Dya46,Dya47,Dya48,Kaw49,Kaw50, Kaw51,Kaw52,Sal53,Sal54,Sal55,Sal56,Sal57,Max58,Max59,Max60,Mal61,Mal62,Mal63,Sh64,Sh65,Sh66,Sh67,G ue68,Gue69,Gue70,Gue71) and after a week of incubation on a particular medium (PVK), a halo zone formed surrounding each colony, indicating their notable zinc solubilizing capabilities. Variations in the growth and appearance of the zinc-solubilizing bacterial isolate were observed in the soils.The soil samples from Dibaga, Qushtapa, Khalifan, and Maxmur had the lowest ZSB populations, whereas the soil samples from Choman and Rawandz had the largest.

It was discovered that the diameters and lengths of these isolated strains were quite comparable. Every isolate was aerobic, gram positive, and gram negative. On an agar plate, their colony looked spherical and creamy, about 27 isolates according to Bacillus subtillus ,23 isolate according to Delftia tsuruhantnsis and 21 isolate Pseudoxanyhomonas maxicans of 71 isolates were rod shape, some of genera spore formed and some genera did not exhibit motility or spore formation, and all isolates responded well to catalase and oxidase. They were able to cultivate at 37˚C but not at 4˚C or 44˚C, but some genera they showed negative response to gelatin and some genera they showed optimistic response to gelatin and urease some genera they showed negative response to urease and all isolates showed positive response to nitrate reduced nitrite. A bacterial strain was isolated utilizing Bergey's manual of determinative bacteriology, morphological analysis, culture, and other biochemical analyses are *Bacillus subtilis, Delftia tsuruhatnsis and psudoxanthomonas Mexicans* [20].

3.2. Zinc Solubilizing Activities

The Z-solubilizing efficacy of each isolated ZSB strain was evaluated on solid PVK media. On agar plates, the formation of a distinct halo zone signifies Z-solubilizing activity. It can be inferred that these strains have the ability to solubilize zincs because they produce a clear zone. All isolates have the capacity to solubilize zinc, as shown in results table (1) and picture (4). Significantly distinct from other isolates, isolate T19 had the highest Z-solubilizing activity, belonging to Bacillus subtilis, at 77.777%, while isolate T18 treatment had the lowest Z-solubilizing activity, belonging to Delftia tsuruhatnsis, at 34.600%.

The findings are consistent with those of [21] .By chelating the metal with organic compounds produced during their metabolic processes, the zinc solubilizing bacteria (ZSB) use a variety of methods to solubilize zinc. These bacteria help solubilize zinc by producing and excreting organic acids through enzymatic means.

[22], To release zinc from its insoluble forms, zinc-solubilizing bacteria use three primary chemical processes: the synthesis of organic acids, the creation of metal chelators, and the manufacturing of additional compounds. These often include organic acids like gluconic acid, citric acid, hydrochloric acid, oxalic acid, and malic acid.

According to [23] ,Zinc Solubilizing Bacteria (ZSB) use a variety of methods to solubilize zinc, such as chelating the metal with organic compounds created during their metabolic processes. These results are consistent with their findings. These bacteria help solubilize zinc by producing and excreting organic acids enzymatically [24]and [25]). The most successful isolates of Bacillus subtilis, Delftia tsuruhatnsis, and Psudoxanthomonas Mexicans were selected for the majority of experiments based on the aforementioned results.

Table 1. Estimation of Zinc solubilizing efficiency of isolate bacterial strains using pikovaskaya's medium.

Treatments	Locations	Means
T1	Choman	58.800^{abc}
T2	Dibaga	63.000^{ab}
T3	Shaqlawa	58.000^{abc}
T4	HajiOmeran	54.000^{bcd}
T5	Rawandz	64.4250^{ab}
T6	Qushtapa	59.000^{abc}
T7	Grdarasha	45.3333^{bcd}
T8	Galala	55.000^{abc}
T9	Xabat	50.700^{bcd}

T10	Kore	53.5556^{bcd}
T11	Khalifan	47.6667^{bcd}
T12	Alyawa	63.6667^{ab}
T13	Harir	48.2222^{bcd}
T14	Dyana	44.3333^{bcd}
T15	Kawrugosk	63.800^{ab}
T16	Saladdin	58.8333^{abc}
T17	Maxmur	37.8222^{cd}
T18	Malaqara	34.600^{d}
T19	Shamamar	77.777^{a}
T20	Guer	50.7250^{bcd}

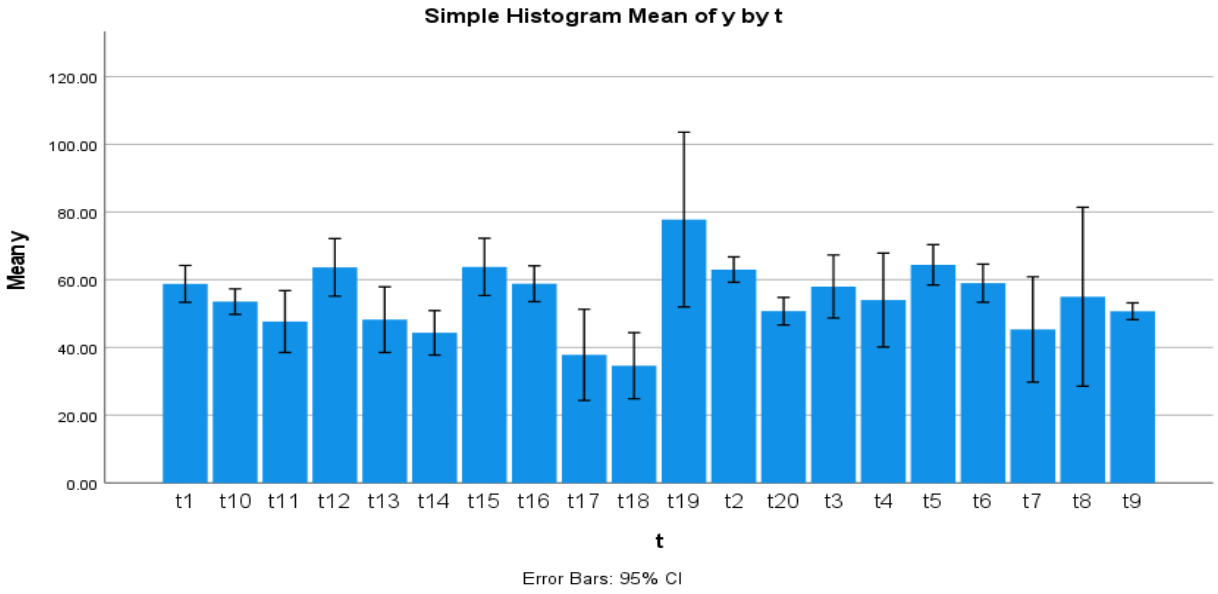


Fig.4 Potential Zn-solubilizing bacteria's Zn solubilization zone (mm). This bar chart presents the mean values of variable y across different treatments or groups labeled t1 to t20. Each bar shows the mean of y for that treatment, and the vertical lines (error bars) signify the 95% confidence intervals (CI) around the means, indicating the reliability of each mean estimate.

Conclusions

This study successfully isolated and identified zinc-solubilizing bacteria (ZSB) from Erbil Governorate soils, highlighting their potential role in enhancing zinc availability in calcareous soils. Several bacterial isolates demonstrated significant zinc solubilization efficiency on Pikovskaya's agar medium, with clear variation among isolates from different locations. Morphological, biochemical, and molecular analyses revealed that the most efficient isolate belonged to genera such as *Bacillus subtilis , Delftia tsuruhatnsis and psudoxanthomonas Mexicans*.The ability of these native bacterialisolate to convert insoluble zinc into bioavailable forms suggests their potential application as eco-friendly biofertilizers to improve plant nutrition and support sustainable agricultural practices. The use of such bioinoculants can reduce dependence on chemical fertilizers, enhance soil fertility, and contribute to better crop yield and food security in zinc-deficient areas like those in Erbil Governorate. Further greenhouse and field trials are recommended to evaluate the practical effectiveness of these isolates under real agricultural conditions.

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عزل وتوصيف البكتيريا المذيبة للزنك في تربة محافظة اربيل

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الخلاصة
الزنك (Zn) عنصر غذائي دقيق أساسي، ضروري للعديد من العمليات الفسيولوجية والكيميائية الحيوية في النباتات، بما في ذلك تنشيط الإنزيمات، وتخليق البروتينات، وتنظيم النمو. ومع ذلك، فإن توافره الحيوي في التربة الجيرية، المنتشرة في العديد من المناطق الزراعية، بما في ذلك محافظة أربيل بالعراق، محدود للغاية. وينشأ هذا القيد بسبب ارتفاع درجة حموضة التربة، وميل الزنك إلى الثبات في صور غير قابلة للذوبان، مما يجعله غير متاح للنباتات. ولمواجهة هذا التحدي، ركزت هذه الدراسة على عزل وتوصيف وتحديد الهوية الجزيئية للبكتيريا المُذيبة للزنك (ZSB) من التربة الزراعية في منطقة أربيل. جُمعت عينات التربة تحديدًا من مناطق الجذور لنباتات مزروعة مختلفة، حيث يكون النشاط الميكروبي مرتفعًا عادةً. وفُحصت العزلات البكتيرية لمعرفة قدرتها على إذابة الزنك باستخدام وسط أجار بيكوفسكايا المضاف إليه أكسيد الزنك (ZnO)، وهو شكل ضعيف الذوبان من الزنك. وقد تم استخلاص وفحص 71 عزلة بكتيرية. من بينها، أظهرت ثلاث عزلات - العصوية الرقيقة، ودلفتيا تسوروهاتنسيس، وسودوكسانثوموناس ميكسيكانا - كفاءةً فائقةً في إذابة الزنك، حيث أنتجت مناطق هالة واضحة بمؤشرات إذابة (SI) أعلى من 2.5. أُجريت عملية التحديد باستخدام تقنيات مورفولوجية وكيميائية حيوية وجزيئية، بما في ذلك تسلسل جينات الرنا الريبوسومي 16S. وأكد التحليل الكمي باستخدام مطيافية الامتصاص الذري (AAS) إذابة كميات كبيرة من الزنك في المزارع السائلة بواسطة هذه السلالات. تشير هذه النتائج إلى أن عزلات ZSB المحددة تتمتع بإمكانيات قوية كسماد حيوي لتعزيز توافر الزنك في الترب التي تعاني من نقص الزنك، مما يُحسّن نمو النباتات وخصوبتها. يمكن أن يُوفر استخدام هذه السلالات المحلية نهجًا صديقًا للبيئة ومستدامًا لزيادة الإنتاجية الزراعية في المنطقة.

الكلمات المفتاحية: البكتريا المذيبة للزنك،بيكوفسكيا اجار ، S rRNA16، Bacillus subtilus، psudoxanthomonas Mexicans و Delftia tsuruhatnsis.