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Molecular Identification of Iron Solubilizing Bacteria in Erbil soil

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ABSTRACT

Iron is an essential micronutrient that plays a fundamental role in various physiological and metabolic functions in plants, such as respiration, photosynthesis, enzyme activity, and chlorophyll synthesis. Although iron is naturally present in most soils, it is often found in insoluble forms, particularly in calcareous or alkaline soils, which significantly limits its availability to plants and leads to iron deficiency, negatively affecting crop yield and plant health. This study was conducted to isolate, characterize, and molecularly identify iron-solubilizing bacteria (ISB) from agricultural soils collected in the Erbil Governorate of Iraq. Soil samples were taken from the rhizosphere zones of cultivated plants and inoculated onto King's B agar medium supplemented with iron oxide (FeO) to detect ISB based on their ability to form clear halos around the colonies, indicating iron solubilization. A total of 114 diverse bacterial isolates were obtained and further characterized using morphological, biochemical, and molecular methods, including 16S rRNA gene sequencing. The isolates were identified as Streptococcus sobrinus (35 strains), Pseudomonas putida (40 strains), and Stenotrophomonas acidaminiphila (39 strains). Among these, two highly efficient isolates, Fe9 and Fe19, demonstrated the highest iron solubilization potential, forming distinct halo zones with solubilization indices (SI) greater than 2.5. Their iron-releasing capacity in liquid culture was also confirmed through precise quantitative analysis using Atomic Absorption Spectroscopy (AAS). The findings suggest that these native, beneficial bacterial strains have strong potential to be developed into environmentally friendly biofertilizers for improving iron availability in soils, thus enhancing plant growth and reducing reliance on synthetic chemical fertilizers in modern sustainable agricultural practices.

***Keywords*:** Biofertilizer; Molecular identification; 16S rRNA sequencing; Iron solubilizing bacteria; Microorganisms that dissolve iron..

INTRODUCTION

Iron-solubilizing bacteria are crucial for increasing iron bioavailability through a variety of biochemical processes since iron is a micronutrient that is necessary for plant growth but is frequently scarce in soil due to its poor solubility. Since iron is a necessary ingredient for plant growth, solubilizing it in soil is a critical step in ensuring that plants can access it. In soil, iron is found in two main oxidation states: ferrous iron (Fe^{2+}) and ferric iron (Fe^{3+}). Fe^{3+} is primarily reduced to Fe^{2+}, which is more soluble in some soil types, in order to solubilize iron. The primary mechanisms in this process are broken down as follows: Iron complexation with soil components, pH and redox conditions, organic acid release, root exudates and phytosiderophores, reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}), and soil microorganisms [1]. For describing these helpful microorganisms from soil habitats, especially those in places like Erbil, Iraq, molecular identification methods like 16S rRNA gene sequencing offer accurate taxonomic resolution [2]. Iron-solubilizing bacteria have a crucial role in enhancing plant nutrient uptake and soil fertility in Erbil, where calcareous conditions frequently cause soils to lack iron [3]. The precise identification and classification of these bacteria from complicated soil communities has been made possible by recent developments in molecular techniques, such as PCR amplification and sequencing of bacterial 16S rRNA genes [4]. By transforming insoluble iron compounds into bioavailable forms, iron-solubilizing bacteria (ISB) support sustainable agriculture by enhancing plant productivity and health [5]. Using molecular techniques like 16S rDNA sequencing to identify these microorganisms guarantees precise classification and exposes the diversity of microbes in particular settings, such as the agricultural soils of Erbil [6]. To precisely identify and categorize bacterial strains with the capacity to solubilize iron from insoluble forms is the goal of the molecular identification of iron solubilizing bacteria (ISB) in Erbil soil. This aids in locating native and effective ISB strains in the nearby soil. Utilizing molecular techniques like 16S rRNA

sequencing, ascertain their taxonomic place and genetic diversity. To increase the amount of iron in the soil, choose suitable candidates to be used as biofertilizers. Reduce the Erbil region's reliance on chemical fertilizers to promote sustainable farming methods.

MATERIALS AND METHODS

2.1. Soil Sample Collections

In September 2024, soil samples were taken from various locations in the Erbil Governorate/Kurdistan region of Iraq, including the following: Gordarasha, Khabat, Choman, Harer, Makhmoor, Qushtapa, Dibaga, Haji Omeran, Kawrgosk, Shaqlawa, Dyana, Kore, Xalifan, massif, Malaqara, Alyawa, Gwer, Rwandz, Shamar, and Galala. The soils were moved to the University of Salahaddin Erbil's Microbiology Laboratory at the College of Agriculture Engineering Science. After being collected in sterile bags and stored at 4°C, the samples were processed a few days later.

2.2. Isolations and Identifications of Bacterial strains:

Following Anderson and Pascual's (2000) procedures, soil samples were serially diluted and then cultivated onto a plate of Biotite containing King's B medium agar (Proteose peptone 20.0, Biotite 2, Dipotassium hydrogen phosphate 1.50, Magnesium sulphate heptahydrate 1.50, Agar 20.0, and Final pH 7.2±0.2 all in gm / Liter) with unsoluble iron compound (FeO) added. In accordance with Bergey's Manual for Determinative Bacteriology, microscopical, morphological, biochemical, and physiological tests were performed following a 75-hour incubation period at 28°C. Cell shape, gram stain, colony morphology, color, size, spore formation, arrangement, motility, colony characters, flagellum observation, aerobic test, pigmentation, oxidase and catalase test, gelatin liquefaction, starch fermentation, hydrolysis, glucose and arginine dihydrolase, levan production, nitrate reduction, utilization of trehalose and tryptophane, growth at 4˚C and 41˚C, and various sugar utilization were among the findings [7] and [8].

2.3. Molecular Identification:

2.3.1. Extraction of genomic DNA from bacterial culture cells

Using the GeneAll® ExgeneTM for Clinic Cell SV small kit (Songpa-gu, Seoul, KOREA), genomic DNA was isolated from pure cultures. Freshly harvested culture cells should be used. During lysis incubation, shaking or vortexing can significantly increase lysis efficiency and shorten the time needed for full lysis.

2.3.2. Estimation of Extracted DNA

Agarose gel electrophoresis is used to estimate the isolated genomic DNA prior to a PCR run. It uses a 0.8% agarose gel and runs for 45 minutes at 85 V [9].

2.3.3. Determination of DNA concentration

After calibrating the device and setting the initial blank to zero absorption, the Nano Drop 1000 spectrophotometer was prepared to take the OD of the DNA sample. An analysis of the concentration and purity of genomic DNA extraction was carried out, using 1 µL of the extracted genomic DNA to determine the concentration and purity. Pure DNA is indicated by a ratio of 1.8 to 2.0, whereas the presence of RNA in the DNA sample is indicated by a rate greater than 2.0. The presence of protein in the isolated genomic DNA is indicated by a fraction less than 1.8.

2.3.4. Primer preparation for PCR

The primer used in this investigation, which is reported in Table (1), was ordered from Macrogen (Korea). It was made by lyophilizing primers (stock solution) and adding the appropriate volume of free nuclease water from the datasheet to make 100 µM. A suitable solution for the PCR reaction was then made with a concentration of 10 µM. Every primer aliquot was stored at -20°C.

Table 1. Primer sequences used for PCR

Target gene	Sequence of the primers (5’ to 3’)	Amplicon size (bp)
16S rRNA	F:5'CGTTGACTGCCGGTGACAAAC'3	372

2.3.5. DNA amplification

Taq DNA polymerase, PCR buffer, deoxynucleotide triphosphates, gel loading dyes, 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.

2.3.6. Protocol of PCR technique

Every gene was subjected to PCR in a reaction volume of 25 µL. As shown in table (2), the master mix tube contains 12.5 µL, 1 µL of forward and reverse primers, 1 µL of DNA template, and 9.5 µL of sterile (D.W.) deionized water.

Table 2. PCR reaction mixture for genes (50 µL)

Components	Volume (µL)
PCR master mix	25
Primer F	1.5
Primer R	1.5
Genomic DNA template	3
PCR grade water	19

2.3.7. PCR technique procedure

DNA amplification for the 16S rRNA gene was carried out at 95˚C for 5 minutes in the heat cycler to guarantee the full denaturation of DNA templates. The amplicon size and PCR conditions for the 16S rRNA gene understudy are as follows for identifying the species. 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.

2.3.8. Agarose gel electrophoresis

A 250 mL conical flask containing 100 mL of 1× TAE buffer was swirled to thoroughly mix the 1.2 g of agarose. In a microwave oven, the mixture melted. Red safe dye was added at a rate of 10 µL/100 mL of agarose gel after it had been allowed to cool somewhat to between 50 and 55°C. The gel was left to solidify at room temperature after the appropriate comb was placed into the tray and the agarose was gradually poured into the tank down to a depth of roughly 1 cm. The gel is placed in the electrophoresis tank with the wells nearest to the cathode end after the comb has been carefully removed. The gel was just submerged after being coated with 1× TAE running buffer. To load the desired DNA samples, 0.2 volumes of loading dye were pipetted up, and the pipette was filled and emptied several times to combine the sample and loading dye. A well was filled with the prepared mixture. The power source was turned on, the gel tank was closed, and the gel operated at five volts per centimeter. After then, the voltage was raised to 75–100 volts, allowing the electrophoresis to continue for an adequate amount of time. The marker loading dye is used to verify the gel's mobility. When the bromophenol blue ran 3/4 of the way down the gel, the flow of the gel stopped. The gel was photographed using a Polaroid photo documentation camera, the current was turned off, and DNA bands were captured by UV illumination at a wavelength of 240–366 nm using a UV transilluminator [10][11].

2.4. Preservations of Bacterial isolates:

For future research, the isolated bacteria's pure colonies were stored at 4 °C on agar slants and at -75 °C with 25% glycerol [12].

2.5. Efficiency of Iron Solubilizing Bacteria in Iron Solubilization in Soil:

Using the broth culture method and plate screening method, the iron solubilizing activity of each isolate was measured. The effectiveness of iron solubilization on King's ager media was assessed for each suspended colony by adding insoluble iron oxide. Assay on plates Modified King's medium was used to inoculate isolated test organisms, which were then cultured for 72 hours at 28 ± 20C. Halo zones surrounding colonies were measured 48 and 72 hours after incubation, and the following formula was used to compute the iron solubilization index and solubilization efficiency [13]:Solubilization Index, or S.I. S.I. is equal to colony diameter / halo zone diameter. The following formula was used to calculate solubilization efficiency: Effectiveness Solubilization (S.E.) S.E. is equal to colony diameter × 100 halo zone diameter.

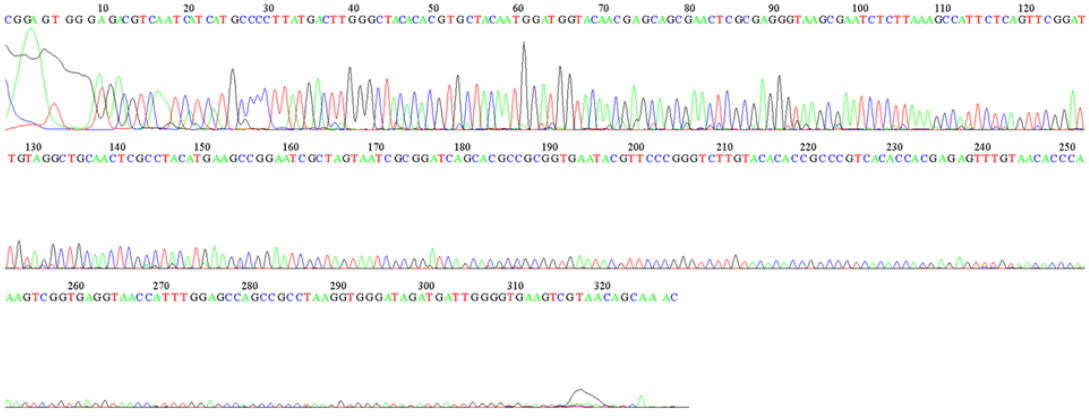
2.6. Statistical Analysis

Using SPSS 16.0, Duncan's H.S.D. multiple range tests were used in every instance to compare treatment means [14].

RESULT AND DISSCUSION:

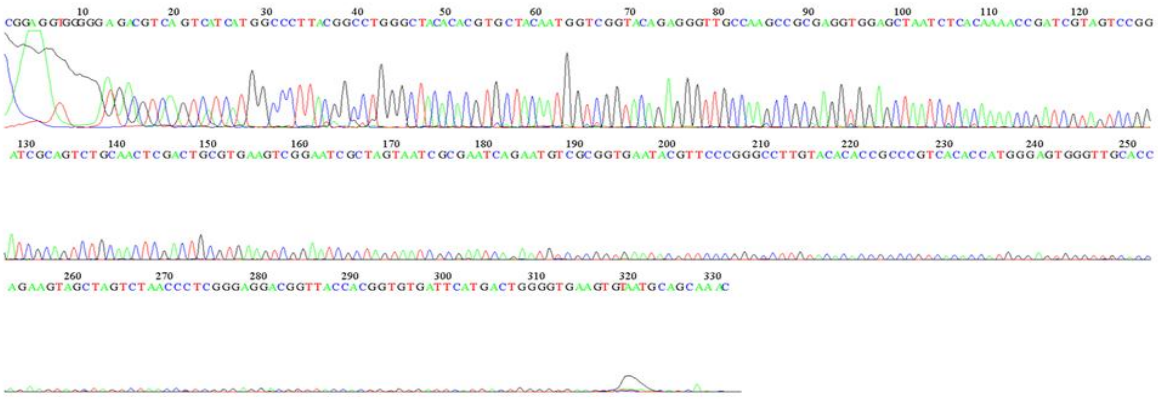
In the Erbil Governorate/Kurdistan region, 114 isolates of iron-solubilizing bacteria were collected and examined from different rhizosphere soil locations.Haj1, Haj2, Haj3, Haj4, Haj5, Haj6, Haj7, Ch8, Ch9, Ch10, CH11, Ch12, Ch13, Ch14, and Ch15, IraqDib16,Dib17,Dib18,Dib19,Dib20,Dib21,Dib22,Dib23,Dib24,Kaw25,Kaw26,Kaw27,Kaw28,Kaw29,Shaq30,Shaq31,Shaq32,Grda33,Grda34,Grda35,Grda36,Grda37,Grda38,Qush39,Qush40,Qush41,Qush42,Qush43,Qush44,Dya45,Dya46,Dya47,Dya48,Dya49,Dya50,Ko51,Ko52,Ko53,Xab54,Xab55,Xab56,Xab57,Xab58,Xab59,Xali60,Xali61,Xali62,Xali63,Xali64,Xali65,Mas66,Mas67,Mas68,Mas69,Mas70,Mas71,Mala72,Mala73,Mala74,Aly75,Aly76,Aly77,Aly78,Aly79,Har80,Har81,Har82,Har83,Har84,Har85,Gwe86,Gwe87,Gwe88,Gwe89,Gwe90,Gwe91,Gwe92,Gwe93,Gwe94,Max95,Max96,Max97Max98,Max99,Max100,Rwa101,Rwa102,Rwa103,Rwa104,Sham105,Sham106,Sham107,Gala108,Gala109,Gala110,Gala111,Gala112,Gala113,Gala114). Iron solubilizing bacteria from various Erbil city areas. Based on their similarity in morphological and biochemical testing, isolates from twenty soil samples were placed on biotite containing King's B medium. Gram-negative isolates with a rod-shaped, greenish yellow colony that is motile and does not produce spores Biochemical analysis of the isolates revealed that they could make catalase, oxidase was positive, while urea and nitrate were negative [15]. showed that a large number of isolates were from the genus Pseudomonas putida, that the isolates were cocci-shaped, yellowish colonies, non-motile, non-spore forming, and positive to the gram response. The isolates' capacity to generate catalase, oxidase, ureas, and nitrate was assessed biochemically. showed that many isolates were of the genus Streptococcus Sobrinus, and that many isolates were of the genus Stenotrophomonas acidaminiphila. The isolates were rod-shaped, yellow or light colony, motile, and non-spore former. They were biochemically characterized for their capacity to produce catalase (positive), oxidase, ureas, and nitrate, which is negative. utilizing techniques outlined in Bergey's Manual of Determinative Bacteriology.

Streptococcus SP.



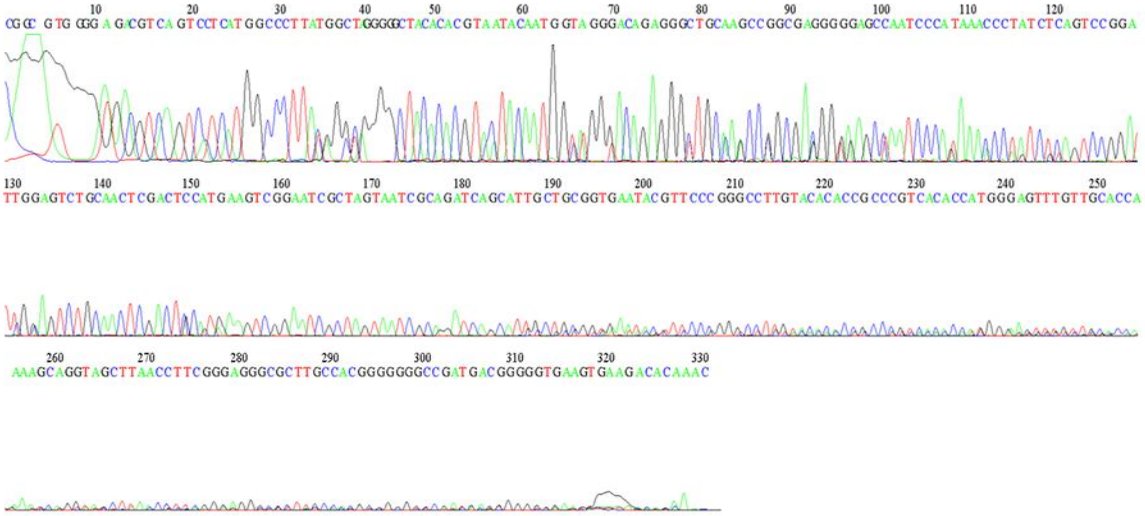
CGGAGTGGGAGACGTCAATCATCATGCCCCTTATGACTTGGGCTACACAC
GTGCTACAATGGATGGTACAACGAGCAGCGAACTCGCGAGGGTAAGCGAA
TCTCTTAAAGCCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACAT
GAAGCCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGT
TCCCGGGTCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACC
CAAAGTCGGTGAGGTAACCATTTGGAGCCAGCCGCCTAAGGTGGGATAGA
TGATTGGGGTGAAGTCGTAACAGCAAAC

Pseudomonas putida



CGGAGGTGGGGGAGACGTCAGTCATCATGGCCCTTACGGCCTGGGCTACA
CACGTGCTACAATGGTCGGTACAGAGGGTTGCCAAGCCGCGAGGTGGAGC
TAATCTCACAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTG
CGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATA
CGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGC
ACCAGAAGTAGCTAGTCTAACCCTCGGGAGGACGGTTACCACGGTGTGAT
TCATGACTGGGGTGAAGTGTAATGCAGCAAAC

Streptophomonas



CGGCGTGGGGAGACGTCAGTCCTCATGGCCCTTATGGCTAGGGGGCTACA
CACGTAATACAATGGTAGGGACAGAGGGCTGCAAGCCGGCGAGGGGGAGC
CAATCCCATAAACCCTATCTCAGTCCGGATTGGAGTCTGCAACTCGACTC
CATGAAGTCGGAATCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAAT
ACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTTG
CACCAAAAGCAGGTAGCTTAACCTTCGGGAGGGCGCTTGCCACGGGGGGG
CCGATGACGGGGGTGAAGTGAAGACACAAAC

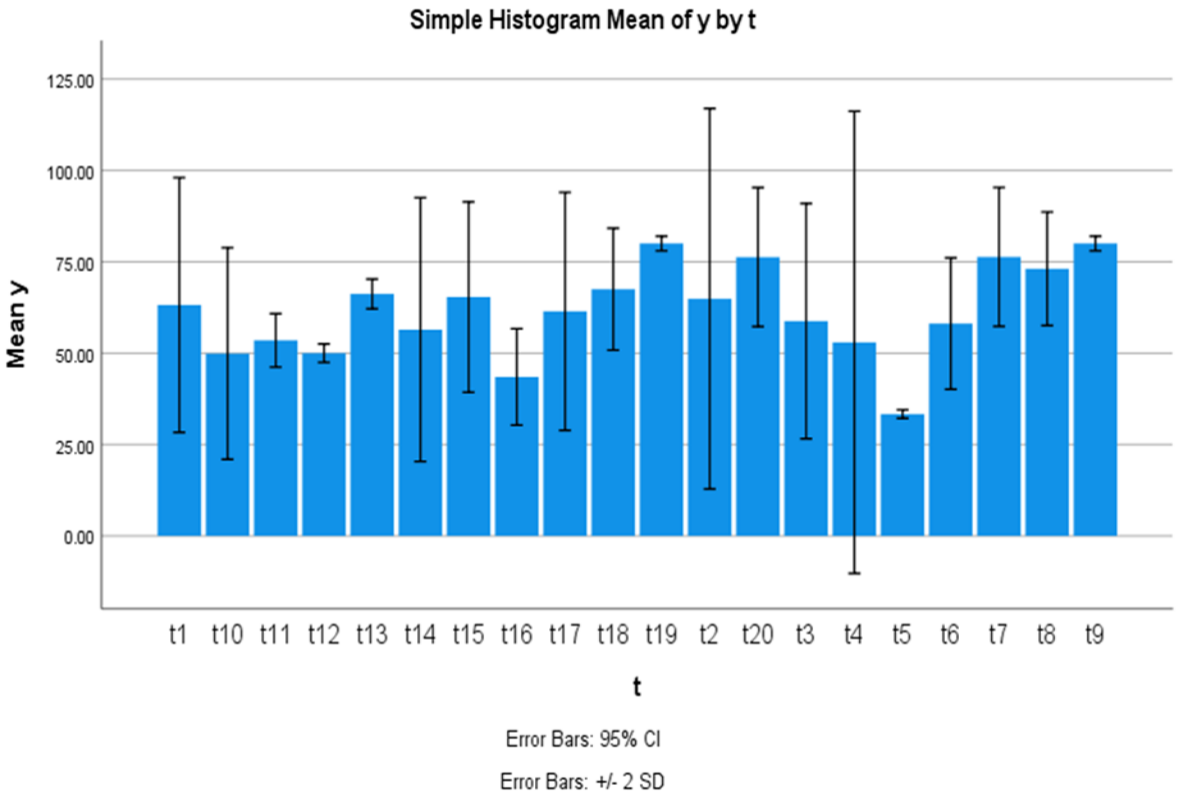
This table displays Twenty treatments (T1 through T20) are used in the experiment, and each one represents a distinct location. For every treatment, the average efficiency values of the microorganisms that dissolve iron are given. Significant variations between the means are indicated by the different superscript letters (a, b, c, and d) that indicate statistical groupings. These groupings are probably the result of a post-hoc test like Duncan's test. Highest Efficiency: Both T9 (Koré) and T19 (Shamamar) belong to group "a" and have the highest mean of 80.0000, which means they

are far higher than many others. Highly effective bacterial strains that solubilize iron may be present in certain areas. Lowest Efficiency: T5 (Shaqlawa), which is in group "d," has the lowest mean (33.3333), which is far lower than that of the majority of places. Intermediate Groups: A large number of sites (such as ab, bc, and bcd) belong to the intermediate groups, indicating a moderate solubility of iron. For instance:T13 (Malaqara): 66.2000 (bc), T7 (Qushtapa): 76.3444 (ab), T8 (Dyana): 73.1000 (ab), and T20 (Galala): 76.2889 (ab).

King's B medium is used to estimate the isolated bacterial strains' iron solubilization efficiency.

Efficiency of iron solubilizing bacteria

Treatment	Location	Mean
T1	Haji omeran	63.1750^{abc}
T2	Choman	64.8889^{abc}
T3	Dibaga	58.7583^{abcd}
T4	Kawrgosk	52.9750^{abcd}
T5	Shaqlawa	33.3333^{d}
T6	Grdarasha	58.1000^{abcd}
T7	Qushtapa	76.3444^{ab}
T8	Dyana	73.1000^{ab}
T9	Kore	80.0000^{a}
T10	Xabat	49.9000^{bcd}
T11	Xalifan	53.4167^{abcd}
T12	Massif	50.0000^{bcd}
T13	Malaqara	66.2000^{abc}
T14	Alyawa	56.4444^{abcd}
T15	Harir	65.3444^{abc}
T16	Gwer	43.5000^{cd}
T17	Maxmur	61.4516^{abcd}
T18	Rwandz	67.5000^{abc}
T19	Shamamar	80.0000^{a}
T20	Galala	76.2889^{ab}



Conclusion:

Microbial isolation, biochemical testing, and molecular (commonly 16S rRNA) analysis would normally form the basis of a study on the molecular identification of iron-solubilizing bacteria in Erbil soil. Significant promise has been demonstrated by local iron-solubilizing bacterial isolates from Erbil's agricultural soils for the solubilization of insoluble iron complexes. Clear halo formation and high solubilization indices indicated that the isolates, especially Streptococcus sobrinus, Pseudomonas putida, and Stenotrophomonas acidaminiphila, had effectively dissolved iron. Their authenticity was verified by molecular identification, and their effectiveness in liquid culture was established by AAS analysis. According to these results, these bacterial strains may be successfully employed in the creation of iron biofertilizers to promote plant growth.

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التعرف الجزيئي على بكتيريا ذائبة للحديد في تربة أربيل

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الخلاصة

الحديد هو مغذٍّ دقيق أساسي يلعب دورًا أساسيًا في وظائف فسيولوجية واستقلابية مختلفة في النباتات، مثل التنفس، والتمثيل الضوئي، ونشاط الإنزيمات، وتخليق الكلوروفيل. على الرغم من أن الحديد موجود بشكل طبيعي في معظم الترب، إلا أنه غالبًا ما يكون موجودًا في أشكال غير قابلة للذوبان، لا سيما في الترب الكلسية أو القلوية، مما يحد بشكل كبير من توفره للنباتات ويؤدي إلى نقص الحديد، مما يؤثر سلبًا على غلة المحاصيل وصحة النباتات. تم إجراء هذه الدراسة لعزل وتصنيف وتحديد بكتيريا إذابة الحديد ***(ISB)*** من الترب الزراعية التي تم جمعها في محافظة أربيل في العراق. تم أخذ عينات من التربة من مناطق الجذور للنباتات المزروعة وزرعها في وسط أجار كينغ المقوى بأكسيد الحديد ***(FeO)*** لاكتشاف ***ISB*** بناءً على قدرتها على تشكيل هالات واضحة حول المستعمرات، مما يشير إلى إذابة الحديد. تم الحصول على ما مجموعه 114 عزلة بكتيرية متنوعة وتم تصنيفها بشكل أكبر. باستخدام الطرق المورفولوجية والبيوكيميائية والجزيئية، بما في ذلك تسلسل جين 16 ***S rRNA.*** تم تحديد العزلات على أنها ستربتوكوكوس سوبريش (35 سلالة)، وزرّلاودوموناس بوتيدا (40 سلالة)، وستينوتروفيوموناس أسيداميني فيلا (39 سلالة). من بين هذه، أظهرت عزلاتان ذات كفاءة عالية، ***Fe9*** و***Fe19***، أعلى إمكانيات إذابة الحديد، حيث شكلت مناطق هالة مميزة مع مؤشرات إذابة ***(SI)*** أكبر من 2.5. تم تأكيد قدرتها على إطلاق الحديد في الثقافة السائلة أيضاً من خلال تحليل كمي دقيق باستخدام الطيفية الامتصاص الذري ***(AAS).*** تشير النتائج إلى أن هذه السلالات البكتيرية الأصلية والمفيدة لديها إمكانيات قوية لتطويرها إلى سماد حيوي صديق للبيئة لزيادة توفر الحديد في التربة، وبالتالي تعزيز نمو النبات وتقليل الاعتماد على الأسمدة الكيميائية الاصطناعية في ممارسات الزراعة المستدامة الحديثة.

الكلمات المفتاحية: الأسمدة الحيوية؛ التعرف الجزيئي؛ تحديد جزيئي تسلسل 16 Sالر RNA؛ الكائنات الحية الدقيقة التي تذوب الحديد.؛. بكتيريا ذائبة للحديد.