Tikrit Journal for Agricultural Sciences (2024) 24 (3): *267-283*
DOI: https://doi.org/10.25130/tjas.24.3.21

IRAQI Academic Scientific Journals
العراقية المجلات الأكاديمية العلمية

ISSN:1813-1646 (Print); 2664-0597 (Online)
Tikrit Journal for Agricultural Sciences
Journal Homepage: http://www.tjas.org
E-mail: tjas@tu.edu.iq

TJAS

Isolation and molecular identification for the first time in Iraq of sulfur-oxidizing bacteria and testing their possession of the plant growth stimulating criteria

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KEY WORDS:
Molecular identification, *Thiobacillus* spp, NCBI, SO_4-Production , P-Solubilizing, IAA, Siderophorses, Gypsipherous Soil

Received: 23/06/2024
Revision: 20/08/2024
Proofreading: 26/08/2024
Accepted: 10/09/2024
Available online: **30/09**/2024

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ABSTRACT

To prepare environmentally friendly bio-fertilizer that enhances the properties of alkaline soils, particularly Gypsipherous soils, and promotes plant growth from pure and molecularly characterized sulfur-oxidizing bacterial isolates, 53 samples were collected on 10/February / 2022 from various agricultural sources and sites, all of the samples were inoculated in a liquid thiosulfate medium. The isolates were then streaked on solid thiosulfate medium and characterized phenotypically based on their Cutural, microscopic, and biochemical properties. Phenotypical characterization results revealed that 8 isolates exhibited the characteristics of Thiobacillus bacteria, They were given the codes B1, B2, B3, B4, B5, B6, B7, B8, and the color of the colonies was between white, creamy to yellow, Convex and smooth Negative to Gram stain, 4 isolates were motile and 4 were non-motile, 4 isolates were fast-growing they include : B1, B2, B3, B4, like that 4 isolates were slow-growing: B5, B6, B7, B8, all isolates were positive for catalase and oxidase tests. The results showed that all isolates were efficient in: Lowering the pH of the medium after 21 days of incubation, phosphate Solubilizing, Producing indole acetic acid (IAA), sulfates, as isolate B3 outperformed all isolates in these properties and recorded 4.52, 91.904 mg P l^{-1}, 21.2 µg ml^{-1}, 1.754% respectively and gave a heavy growth on chelating compounds medium, isolate B7 gave the lowest values, recording 7.06, 35.101 mg P L^{-1}, 11.9 µg mL^{-1}, and 0.884 and gave a weak growth on chelating compounds medium, Isolates that demonstrated efficient growth and possessed the criteria for promoting plant growth molecular diagnostic result for the four isolates :B1,B2,B3and B4 isolates based on the amplification of the 16S rRNA gene. The nucleotide sequences were then analyzed and compared to those of global strains listed on the (NCBI) website. The results showed that isolates B1, B2, B3, and B4 had 99.26%, 99.26%, 99.23%, and 99.17% similarity to *Thiobacillus thioparus* strain Starkey, *Thiobacillus denitrificans* strain DSM 739, *Thiobacillus denitrificans* strain NCIMB 9548, and *Thiobacillus thiophilus* strain D24TN, respectively. The isolates were assigned the accession numbers ON844981, ON844984, ON844983, and ON844982, respectively, in the National Center for Biotechnology Information (NCBI) data base. This molecular diagnosis and registration in the NCBI is the first in Iraq.

العزل والتشخيص الجزيئي لأول مرة في العراق للبكتريا المؤكسدة للكبريت واختبار امتلاكها للمعايير التشجيعية لنمو النبات

عبدالكريم عريبي سبع الكرطاني وشيماء عارف سرحان
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الخلاصة

بهدف تحضير لقاح حيوي صديق للبيئة يحسن من صفات التربة القاعدية ولاسيما الجبسية ويشجع نمو النبات من عزلات بكتيرية مؤكسدة للكبريت نقية ومشخصة جزيئياً، جُمِعَت 53عينة بتاريخ 2022/2/10 م من مصادر ومواقع زراعية مختلفة، زُرعت العينات في وسط الثيوسلفات السائل، ثم تم تنقية العزلات على وسط الثيوسلفات الصلب وشُخصت العزلات مظهريا بالاعتماد على صفاتها المزرعية والمجهرية والكيموحيوية ،اظهرت نتائج التشخيصات المظهرية والكيموحيوية بان 8 عزلات اعطت صفات بكتيريا Thiobacillus وتم اعطاءها الرموز B1 ،B2 ،B3 ،B4 ،B5 ،B6 ،B7 ،B8 وكان لون المستعمرات بين الابيض والكريمي والاصفر ، محدبة وملساء ، سالبة لصبغة كرام ، 4 متحركة و4 غير متحركة ، 4 سريعة النمو وتشمل B4,B3,B2,B1 ، كذلك 4 بطيئة النمو وتشمل B8,B7,B6,B5 ، جميعها موجبة لاختبار الكاتليز والاوكسيديز ، وبينت النتائج بأن جميع العزلات أبدت كفاءتها في خفض pH الوسط بعد 21 يوما من التحضين وإذابة الفوسفات وإنتاج أندول حامض الخليك IAA ، والكبريتات والمركبات الخالبة إذ تفوقت العزلة B3 على جميع العزلات في هذه المعايير وسجلت4.52 ، 91.904 ملغمP لتر$^{-1}$، 21.2 مايكروغرام مل$^{-1}$، 1.754% على التتابع، واعطت العزلة B7 اقل القيم اذ سجل 7.06 ،35.101 ملغمP لتر$^{-1}$ ، 11.9 مايكروغرام مل$^{-1}$، 0.884 % ، فضلا عن المركبات الخالبة اذ كانت العزلات B5,B4,B3,B1، عالية B6,B2 متوسطة والعزلتين B8,B7 ضعيفة في انتاج المركبات الخالبة ، واختيرت العزلات الكفوءة في النمو و امتلاكها للمعايير التشجيعية لنمو النبات ، وشُخصت جزيئيا بالاعتماد على تضخيم الجين 16SrRNA، ثم تم تحليل تتابع القواعد النتروجينية وعند اجراء التطابق مع السلالات العالمية المدرجة في موقع المركز الوطني للمعلومات التقنية الحيويةNCBI ، تبين النتائج بأن هناك تشابه بنسبة (99.17, 99.23, 99.26, 99.26)% للعزلات B1,B2,B3,B4, مع *Thiobacillus thioparus strain Starkey* ، *Thiobacillus denitrificans strain DSM* ، *Thiobacillus denitrificans strain NCIMB 9548* ، *739 Thiobacillus thiophilus strain D24TN* وسجلت بالارقام ON844981 ، ON844984 ، ON844983 ، ON844982 على التتابع في المركز الوطني للمعلومات التقنية الحيويةNCBI. ويعدهذا التشخيص الجزيئي والتسجيل في NCBI الأول في العراق.

الكلمات المفتاحية : التشخيص الجزيئي ، *Thiobacillus* spp، المركز الوطني للمعلومات التقنية الحيويةNCBI ، انتاج الكبريتات ،اذابة الفوسفات ، اندول حامض الخليك، المركبات الخالبة ، الترب الجبسية .

INTRODUCTION

The soils of dry and semi-arid regions, especially Gypsipherous soils, are characterized by their low content of clay and organic matter and their high content of Gypsum and lime, this has led to the emergence of physical, chemical, fertility, and biological problems, including: Low water retention , high pH, Low nutrient content, Low plant growth promoting Rhizosphere (PGPR) content (Abd and Alkurtany, 2023) especially sulfur-oxidizing bacteria. Sulfur-oxidizing bacteria, Thiobcillus spp., are among the PGPR organisms, as they biologically oxidize sulfur and produce sulfuric acid, which leads to lowering the pH ,and thus increasing the availability of macro- and micronutrients ,as well as dissolving phosphate and producing basic plant hormones such as indole acetic acid (IAA) and chelating compounds(Rana et al.,2020),(Nadeem *et al.*, 2023) .

Sulfur-oxidizing bacteria are characterized as obligate aerobic chemoautotrophic bacteria, as they obtain their energy from the oxidation of sulfur and their carbon from carbon dioxide. Thiobacillus bacteria belong to the Thiobacillacaea family, which is a large group that has the ability to oxidize sulfur compounds and can live at a pH of (10.5-1). (Priyanka *et al.,* 2014). It is belong the genus Thiobacillus and divided according on the pH at which they grow, including *T.thiooxidans , T.copraliticus, T.denitrificans, T.thioparus , T.ferrooxidans, T.novellus* . It was found that the optimal pH for both *T.copraliticus and T.ferrooxidans* is usually between (3-2), while other Thiobacillus species, favor conditions closer to neutrality or even slightly alkaline conditions. All of these species are obligately aerobic, with the exception of *T.denitrificans*. Which can use nitrate as a final acceptor of electrons in anaerobic conditions (Sylvia *et al,* 2005). Diagnosis based on the 16SrRNA gene is of great importance for learning the bacterial species and the percentage of similarity between them and the diagnosed isolates registered in the NCBI Global Gene Bank with high accuracy, and a sufficient indicator for learning the characteristics of the diagnosed bacteria in preparation for dealing with them.(Ali *et al.,* 2023; Ibáñez et al.,2023).

The study aimed to isolate, phenotypic Diagnostic, and evaluate the efficiency of Thiobacillus strains in producing (IAA), chelating compounds, solubilizing phosphorus, producing sulfates, and lowering pH to select the efficient isolates and Molecular identification of them for use in producing a biofertilizer that contributes to improving alkaline soil properties, especiallyGypsipherous soils, increases the availability of nutrients, and encourages growth the plant.

MATERIALS AND METHODS

53 samples were collected on 2/10/2022 from various agricultural sources, the samples included rhizosphere soils of various plants, and mineral water samples from sulfur springs and areas of Salah al-Din and Nineveh Governorate andCompost samples made locally from plant remains. The samples were transferred to the laboratory for isolation and diagnosis.

Isolation and purification of sulfur-oxidizing bacteria(Thiobacillus):

Sulfur-oxidizing bacteria were isolated by inoculating liquid thiosulfate medium with 1 g of soil samples, organic matter, compost, or 1 ml of mineral water samples under sterile conditions, then incubated in a shaking incubator Labtech type, Korean origin for 21 days, according to the method mentioned by(Vidyalakshmi and Sridar, 2007; Suparjo *et al.,* 2019), then the growing isolates were purified by growing them using the straking method on solid thiosulfate medium, and the isolates were preserved on Nutrient Agar Slant medium and placed in the refrigerator at a temperature of 4°C, taking into account renewal every three months throughout the duration of the research, according to what was mentioned. (Aneja, 2005) .

Testing the efficiency of isolates: Test were includes SO_4, indole acetic acid , Sidrophorses Production, the methood condected according to the method in (Payne ,1980; Glick and Patten, 2002; Ryan *et al.*, 2003) respectively .

pH reduction test: The pH of the liquid medium is measured after filtration using a pH device, Hana type, Korean origin

Testing the efficiency of isolates in dissolving phosphate in liquid culture medium (quantitative method): The efficiency of the isolates in dissolving phosphate was estimated quantitatively according to the Pikovskaya liquid method (Pikovskaya , 1948).

Phenotypic diagnosis: Isolats were diagnosed phenotypically based on the cultural, microscopic, and biochemical characteristics of the bacterial isolates: shape, texture, consistency, convexity, transparency, growth on MacConkey medium, and movement by stabbing method, according to (Collins *et al.*, 1995). Microscopic characteristics, Gram stain, biochemical characteristics, and catalase and oxidase tests, based on the methods mentioned in (Collee et al. 1996) .

Molecular identification: The bacterial isolates that showed the highest ability to produce sulfates and biological activities at the level of other isolates were identified according to the method based on analysis of the nucleotide sequence of the 16SrRNA gene.

Genomic DNA Isolation: Genomic DNA was extracted from swab (100) mg of freshly growing pure bacteria colony was used (24 hours) and the genomic DNA was extracted using the ready-made kit for DNA extraction ZR Fungal / Bacterial / Yeast DNA Mini Prep TM prepared by the American company ZR. Genomic DNA was extracted instructions the processing company.

Polymerase Chain Reaction (PCR): The 16S rRNA gene is duplicated after the extraction process using PCR technology with a pair of primers, and the Universal Primer whose sequence is shown in Table (1) to duplicate the region of the ITS region of the selected isolates (Miller *et al.*, 2013) and prepared from (Integrated DNA Technologies company, Canada). A device was used Applied Biosystem Gene-amp PCR System 9700 in the process of replicating the aforementioned gene.

Table (2) Primer preparation and standard reaction conditions: The primers for the study genes were prepared by Macrogen in a freeze-dried form. Then the primers were dissolved in deionized water to give a final concentration of 100 pmol (microlitres) to form the stock solution. The working solution for all primers was prepared by adding 10 microlitres. From the stock solution stored in the freezer at (-20 °C) to (90) microliters of ion-free water to obtain the effective working solution with a concentration of 1-10 picomoles, and the reaction was carried out with a final volume of 100 microliters**.**

Detection of genes: Prepare a polymerase chain reaction (PCR) mixture for the 16S rRNA study genes with a total reaction volume of 25 microliters per box, according to Table (3).

Table 1: Primers used in the study and their sequences

Primer	Sequence	Primer sequence	Tm (°C)	GC%	Size of Product (bp
16s	F	5'- AGAGTTTGATCCTGGCTCAG- 3'	54.3	50.0	1250
RNA	R	5'- GGTTACCTTGTTACGACTT- 3’	49.4	42.1	basepair

Table(2): Primer preparation and standard reaction conditions

No.	Phase	Tm (°C)	Time	No. of cycle
1-	Initial Denaturation	95°C	5 min	1 cycle
2-	Denaturation -2	95°C	45 Sec	
3-	Annealing	56°C	45Sec	35 cycle
4-	Extension-1	72°C	1min	
5-	Extension -2	72°C	5 min.	1 cycle

Table (3): Reaction components of PCR

Component	25 µL (Final volume)
Master Mix or GoTaq® Green Master Mix	12.5µl
Forward primer	10 picomols/µl (1 µl)
Reverse primer	10 picomols/µl (1 µl)
DNA	1.5µl
Distill water	9µl

Agarose gel electrophoresis: The PCR product was separated using electrophoresis on an agarose gel (1.5)%, then the genomic DNA bands were visualized using UV light at a wavelength of (302) nm after being treated with Intron Korea red stain.

Nucleotide sequence analysis: The nucleotide sequences of the amplified gene were determined by PCR immediately after obtaining the 16SrRNA gene replication product by sending a volume of 25 µl of the PCR product and a volume of 10 µl (concentration of 10 pmol) of each primer to the Korean company Bioneer using the global website of the National Center for Technical Information. National Center Biotechnology Information (NCBI) within the Blast sub-window, then the secondary sub-window Nucleotide blast was chosen. The alignment and matching process was performed with the strains registered in the World Gene Bank (alignment file).

BiotechnologyL ab (the device used was Applied Biosystem 3730XL, DNA Sequencer) and the results were compared using a computer program connected to the Internet (Basic Local Alignment Search Tool (BLAST) with the database on the National Center for Biotechnology Information (NCBI), which is carried out by matching the nucleotide sequences of the 16S rRNA gene to the bacterial isolates subject to the research and knowing their type according to the match in the previously mentioned database. After completing the diagnosis of the bacterial

isolates, the percentage of similarity with the registered international strains was recorded, and four Iraqi isolates were recorded in the genetic bank .

RESULTS AND DISCUSSION

Table 4 shows the eight isolates belonging to the Thiobacillacaea family, isolated from different types of compost and rhizosphere soils of some plants growing in Gypsipherous Soil, which were identified based on cultural and microscopic characteristics. The isolates grew after being incubated for 4 days at a temperature of 28°C, which were classified as belonging to the Thiobacillacaea family,Thiobacillus genus, all isolates were characterized by the fact that their colonies had circular, irregular, and convex shapes, complete and smooth edges, and white, creamy, and yellow colors. Isolates B1, B2, B3 and B4 were fast-growing, as growth appeared within 24-48 hours, while isolates B5, B6 appeared within 72 hours, and isolates B7 and B8 appeared within 4 days . Gram stain test the results of microscopic examination of all bacterial samples, including 8 isolates, after staining them using the Gram stain, showed that the bacterial cells were pink in color and were shaped like short rods, This proves that they are stain Gram - negative bacteria, this is consistent with what was found by (Suparjo *et al.*, 2019). The results of Table(4) show that Bacterial isolates (B2, B3, B4, B7) were motile in semisolid nutrient agar medium using the stabbing method, and (B1, B5, B6, B8) were non-motile,this resaltof movement wich refer all isolats of *Thiobacillus* agree whith (Kelly and Wood, 2000 ;Sylvia *et al.*, 2005).

Table (4): Some cultural and microscopic characteristics of Thiobacillus bacteria colonies

Bacterial isolates	Growth	Shape	Texture	Color	Convexity	Mucous	Gram stain	Movement
B1	fast	spherical	smooth	wh ite	convex	mucous	negative	immotile
B2	fast	spherical	smooth	creamy	convex	mucous	negative	motile
B3	fast	spherical	smooth	creamy	convex	mucous	negative	motile
B4	fast	spherical	smooth	creamy	convex	mucous	negative	motile
B5	slow	spherical	smooth	wh ite	convex	mucous	negative	immotile
B6	slow	spherical	smooth	wh ite	convex	mucous	negative	immotile
B7	slow	irregular	smooth	yellow	convex	mucous	negative	motile
B8	slow	irregular	smooth	yellow	convex	mucous	negative	immotile

Table(5)shows that the catalase test results were positive for all isolates, meaning that they all have the ability breaking the hydrogen peroxideand releaseing of oxygen gas bubbles,the resalt agree whith(Alkurtany *et al.,* 2022; Ali *et al.,* 2023). As for the oxidase test shown in the same table, the results were positive for all bacterial isolates because it was colore d perple,the resalt agree whith (Alkurtany *et al.,* 2022). Table(5) shows that all bacterial isolates showed good growth in MacConkey medium, which confirms that all isolates are gram negative and non-lactose fermenting because the coloniesdid not turn pink.

Table (5) Some biochemical tests for isolates of Thiobacillus bacteria

Bacterial isolates	MacConkey test	Oxidase test	Catalase tes	Lactose fermentation
B1	+	+	+	-
B2	+	+	+	-
B3	+	+	+	-
B4	+	+	+	-
B5	+	+	+	-
B6	+	+	+	-
B7	+	+	+	-
B8	+	+	+	-

Table(6) shows that isolate B3 outperformed the rest of the isolates in producing sulfate in the medium, reaching 1.754%, followed by isolate B4, which reached 1.733%. As for isolates B5, B2, and B1, the amount of sulfate was recorded(1.505, 1.557, 1.606)%, respectively. Then they were followed by isolates B8 and B6, which had a sulfate percentage estimated at 1.096 and 1.162%, respectively. The isolate that recorded the least sulfate production was isolate B7, which recorded 0.884%. this resalt du to that all isolats sulfur compounds are oxidased to obtain energy they are chemoautotrophic Bacteria, this result agree whith (Al-Zubaidy, 2009; Yong *et al.,* 2010 ; Kumar *et al.,* 2020 ; Amin and Mihoub , 2021; Twible et al.,2024) .

Table (6): Effect of isolates on the percentage of sulfates produced after 21 days of incubation in liquid media

Bacterial isolates	%(SO_4) Product
B1	1.606
B2	1.557
B3	1.754
B4	1.733
B5	1.505
B6	1.162
B7	0.884
B8	1.096

Table (7) shows that there are two stages to the decrease in pH of the medium, a rapid stage during the first week of incubation and a slow stage after 21 days of incubation. This decrease results from the oxidation of sulfur compounds to sulfate, where in the first stage there is rapid oxidation of sulfate followed by slow oxidation. Isolate B3 recorded the highest decrease in pH after a week. of incubation, as it decreased to 4.90 after adjusting the pH of the medium to 8 before incubation, followed by B2, B1, and B4, which recorded a decrease of 5.40, 5.30, and 5.08 respectively**.** It was followed by isolate B6, which recorded a decrease of 6.15. As for isolates B5, B7 and B8, the pH did not decrease during the first week and remained stable at number 8 of incubation. This can be attributed to the speed of the isolates in oxidizing sulfur compounds and producing sulfur after a week of incubation, and the other isolates do not have it. The ability to oxidize after a week of incubation. In the second stage, after 21 days of incubation, isolate B3 excelled, recording a decrease of 4.52, followed by isolate B2, B1, B5, and B4, which recorded 4.93, 4.85, 4.71, and 5.11 respectively, while isolates B8 and B7 recorded a lower decrease than the rest. The isolates recorded 5.95 and 7.06, respectively. This difference is attributed to the different genomic and species of Thiobacillus bacteria and their ability to produce sulfates, which in turn works to lower the pH of the medium. This is consistent with what was found by(Suparjo *et al.*, 2019 ; Hassan and Ajaj, 2021; Twible *et al.*,2024).

Table (7): The effect of sulfur-oxidizing bacteria in reducing the pH of the medium after a week and after 21 days of incubation

Bacterial isolates	pH of the medium before incubation	pH of the medium after one wek	pH of the medium after 21 day
B1	8	5.30	5.11
B2	8	5040	4.71
B3	8	4.90	4.52
B4	8	5.08	4.85
B5	8	8	4.93
B6	8	6.15	5.36
B7	8	8	7.06
B8	8	8	5.95

Figure (1) shows the ability of isolates belonging to the Thiobacillus genus to dissolve mineral phosphorus when inoculated with Pikovskaya liquid medium containing tricalcium phosphate after 7 days of incubation. It is clear from this figure that all isolates showed their efficiency in dissolving phosphate, as isolate B3 excelled. On all isolates, it gave a solubility of phosphorus amounting to 91.904 mg PL^{-1}, followed by isolate B1, which gave a solubility of 84.396 $mgPL^{-1}$, while isolate B4 gave a solubility of 78.218 $mgPL^{-1}$, while isolate B6 gave a solubilization of phosphorus amounting to 74.322 $mgPL^{-1}$. compared to the comparison treatment, which gave 2.498 mg P L^{-1}, followed by isolate B5, which reached 68.049, while isolate B7 gave the least phosphate solubility, reaching 35.101, compared to the rest of the

isolates. The reason for this may be attributed to the release of organic acids that affect the dissolution of phosphorus, The organic acids such as oxalic acid, citric acid and formic acid that chelate calcium, iron and aluminum ions bound to phosphate and then liberate phosphorous and make it more available (Satyaprakash *et al.*, 2017) and this study agrees with the findings of(Dabo et al.,2019; Alkurtany *et al.*, 2022). Mardad et al. (2014) found that glucose is a source of carbon, and ammonium phosphate $(NH_4)_2SO_4$ is a source of nitrogen, which affects the increase in phosphate avelabilty by lowering the pH of the culture medium. Some researchers explained the increase in solubility as a result of the production of mineral acids. This may be attributed to their ability to oxidize sulfur compounds and produce sulfuric acid, which led to a lowering of the pH, as shown in Table 8. In addition, the isolates are capable of producing organic acids, which causes rapid dissolution of phosphate. This result agree with (Alwandawi,2020;Amin and Mihoub,2021; Kumar *et al.*, 2020).

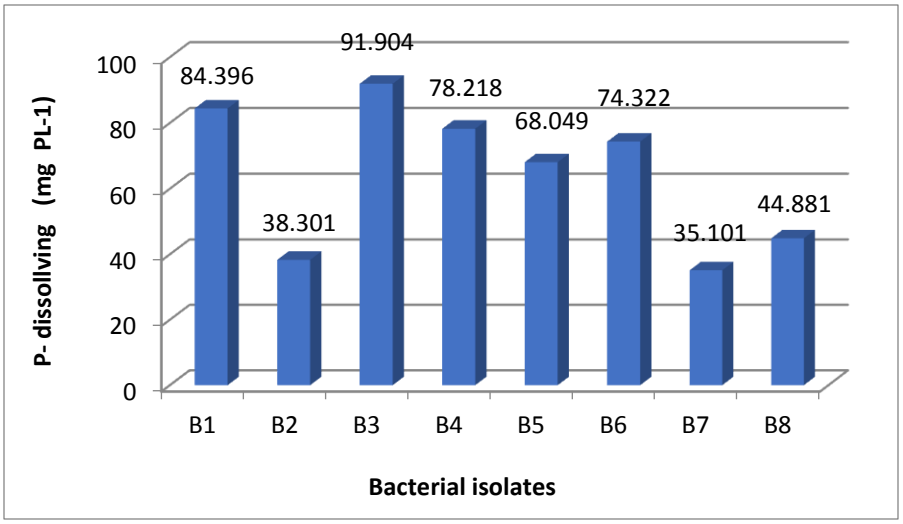


Figure (1): Efficiency of bacterial isolates in dissolving tricalcium phosphate (TCP) mg P L^{-1} in the figure

Figure (2) shows the efficiency of bacterial isolates in producing indole acetic acid. It is clear that isolate B3 excelled in producing indole, giving 21.2 µg ml^{-1}, followed by isolates B4, B5, B8, B1,B2,B7,B6 at rates of (15.2, 14.2, 12.8, 12.8,12,11.9 and 10.9) µg ml^{-1}, respectively, and the lowest indole production was for isolate B6. The reason for the variation by in the amount of indole produced by isolation different species and strains is due to the variation. The genetics of these isolates is that they belong to different species, which is reflected in their biological characteristics, including their secretions in the growth medium (Khalil and Alkurtany, 2018; Alkurtany *et al.*, 2022; Ali *et al.*, 2023).

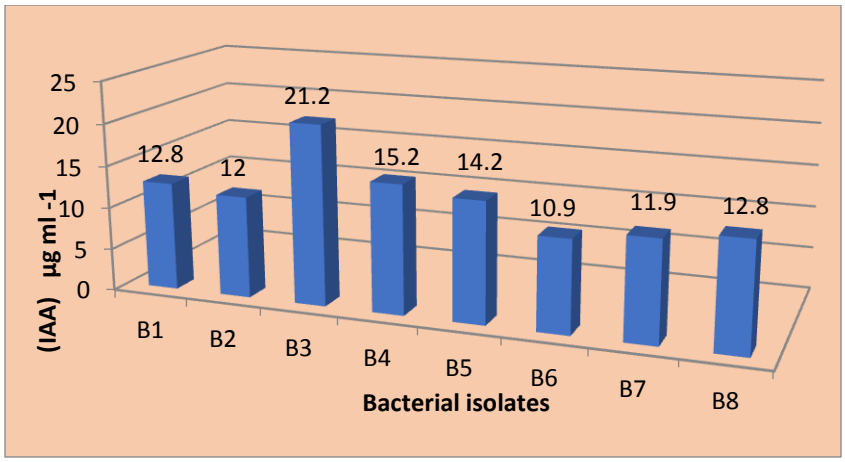


Figure (2): Efficiency of bacterial isolates in producing indole acetic acid (µg ml^{-1}).

Table(8) shows the ability of the isolates to produce iron-chelating compounds, and the results indicate that all bacterial isolates are able to grow on this iron-chelating compounds medium, as isolates (B1, B3, B4, B5) gave a high ability to chelate through their dense growth, while isolates (B2 gave , B6) had a moderate ability to chelate iron, with the exception of the two isolates (B7, B8) which showed a weak ability to chelate iron**.**

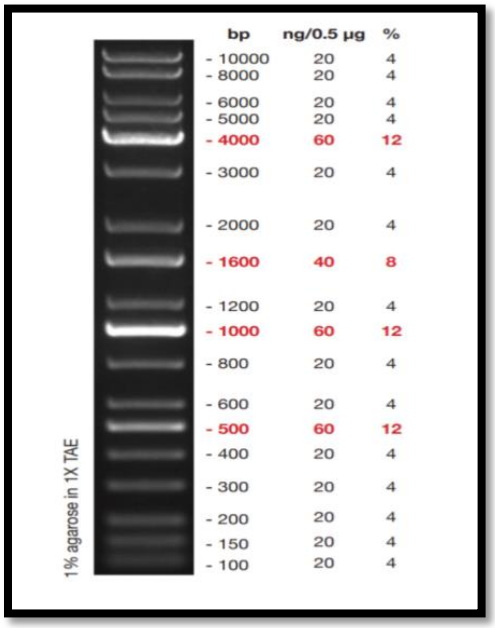
Table (8): The testing ability of Thiobacillus bacteria isolates to produce Siderophorses

Bacterial isolates	iron chelating
B1	+++
B2	++
B3	+++
B4	+++
B5	+++
B6	++
B7	+
B8	+

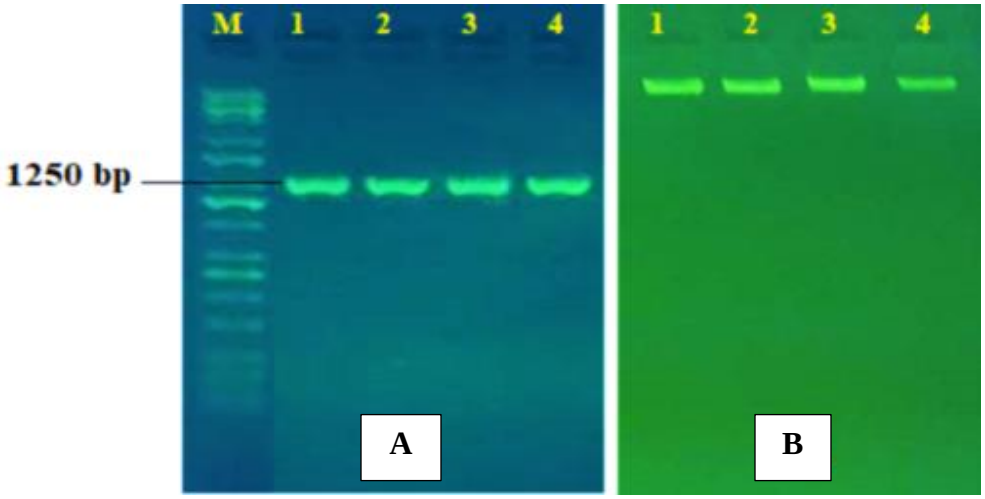
The difference between the isolates in their production of chelating compounds is due to the difference in their genetic makeup and their differences in growth and the amount of iron ey chelating from the medium. Bacteria that do not grow on this medium do not have the ability to produce chelating compounds and cannot withdraw iron from the medium, and the bacteria that produce these compounds have the ability to withdraw The ion from the compound 2,2dipyrdil, binding to it, and transporting it into the bacterial cell to benefit from it in metabolic activities. This result agree with (Leoni *et al.*, 1996; Alkurtany *et al.*, 2023; Ali *et al.*, 2023).

Figure (3) shows the ladder of the DNA fragment used in the amplification process, and Figure (4-B) shows the result of gel electrophoresis to extract the DNA of four isolates and shows the presence of one band for each isolate, which indicates the purity of the DNA and the accuracy of the extraction, and Figure (4- A) shows the result of the PCR polymerase process after amplifying a piece of DNA. The isolates were molecularly characterized using the specialized primer (1250 pb) according to what was mentioned by (Miller *et al.,* 2013), and the results showed the appearance of bands with a size of 1250 base pairs, which is the expected size, as this size is confirmed in a certain way. It is conclusive that the resulting bands are related to bacterial species, and this is evidence of the accuracy of PCR, which targets the ribosomal region of the gene in 16SrRNA, and was sufficient to diagnose it molecularly .(Hassan and Ajaj, 2021 ; Ibáñez et al.,2023).

Table (9) shows the molecular diagnosis of the species and the percentage of similarity between the isolates diagnosed and registered in the Gene Bank with the global isolates registered in NCBI. The isolates showed a similarity of 99.26, 99.26, 99.23, and 99.17% to isolates B1, B2, B3, and B4, respectively with The bacterial strains that were registered at the NCBI International Genetic Center in the numbers mentioned in Table (9), which are the first strains recorded in Iraq, with a match rate of (99.17-99.26)%, they can be considered genetically different isolates because there is no match (100)%, and this can be attributed to the occurrence of many genetic variations due to environmental factors and the environmental pollution factors, which leads to the occurrence of genetic mutations and thus genetic variation occurs which was reflected on the physiological factors of these species (Martins *et al.,* 2013; Cadet and Wagner, 2014; Hassan and Ajaj, 2021). Figures (5,6, 7,8) show the genetic tree of the diagnosed bacteria and show their closeness. With Thiobacillus bacteria listed in Table 9.



Figure(3) : SiZer 100bp DNA ladder (Kapa /USA)



Figure(4-A): PCR product the band size . The product was electrophoresis on 1.5% agarose at 5 volt/cm^2. 1x TBE buffer for 1:30 hours. M: DNA ladder (100)
Figure(4-B): Gel electrophoresis of genomic DNA extraction from bacteria 1% agarose gel at 1houre

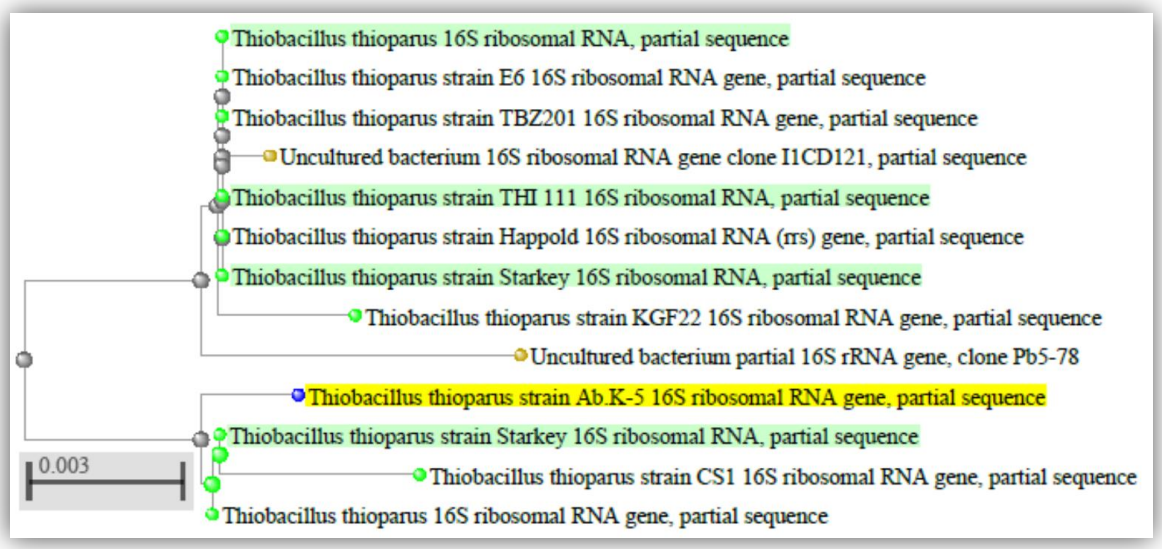


Figure (5): Genetic tree of Thiobacillus thioparus strain Ab.K-5

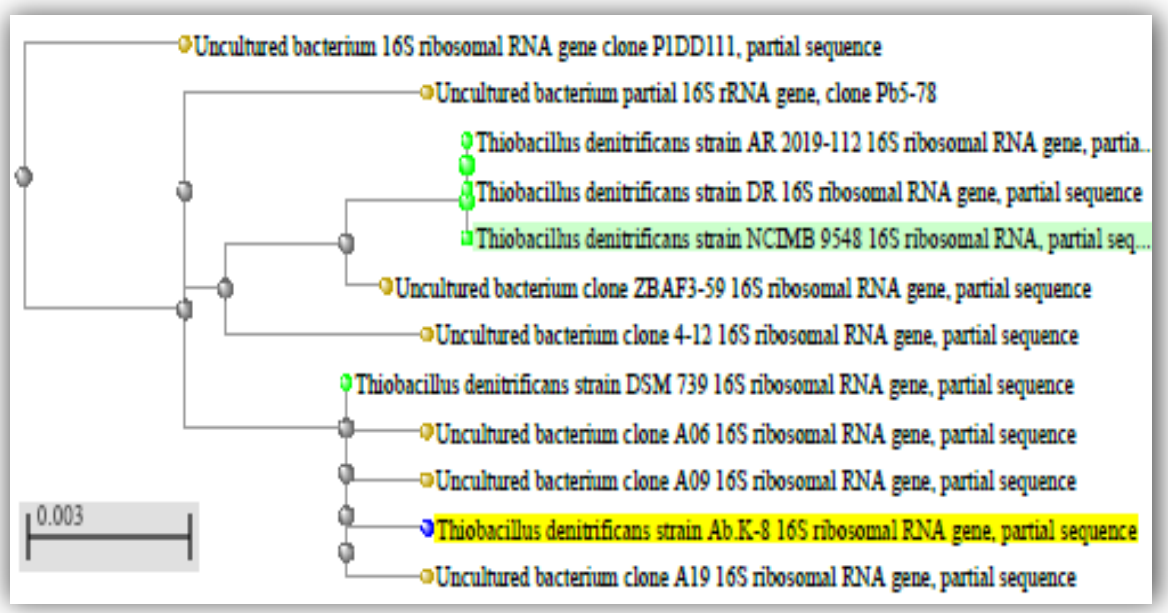


Figure (6): Genetic tree of Thiobacillus denitrificans strain Ab.K-8

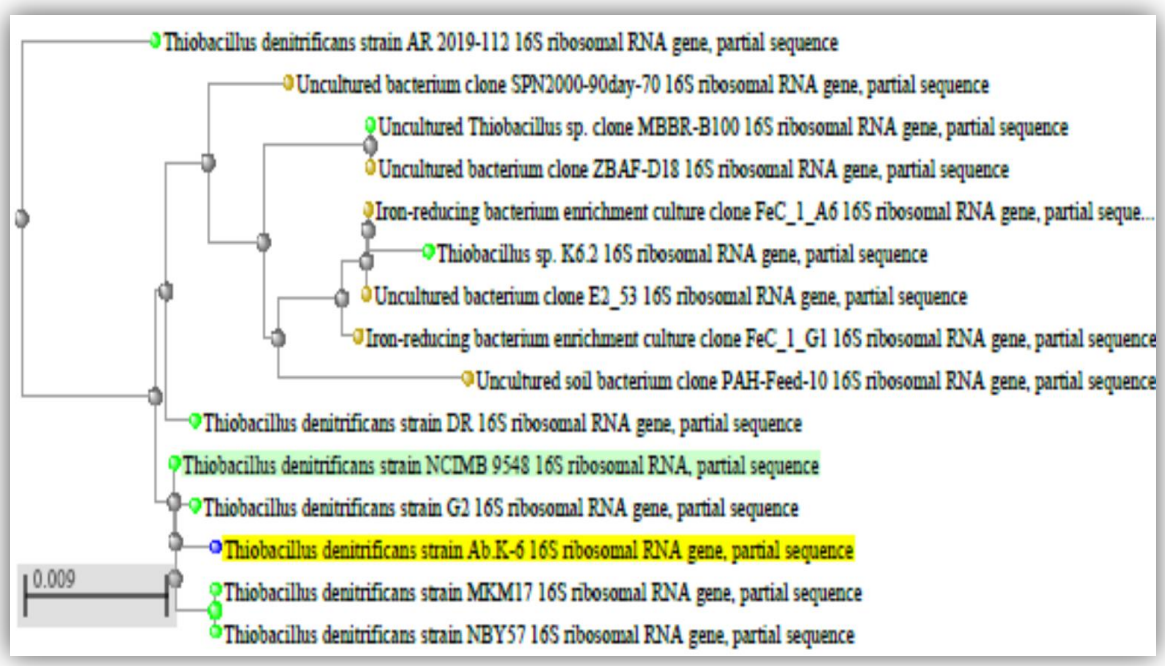


Figure (7): Genetic tree of Thiobacillus denitrificans strain Ab.K-6

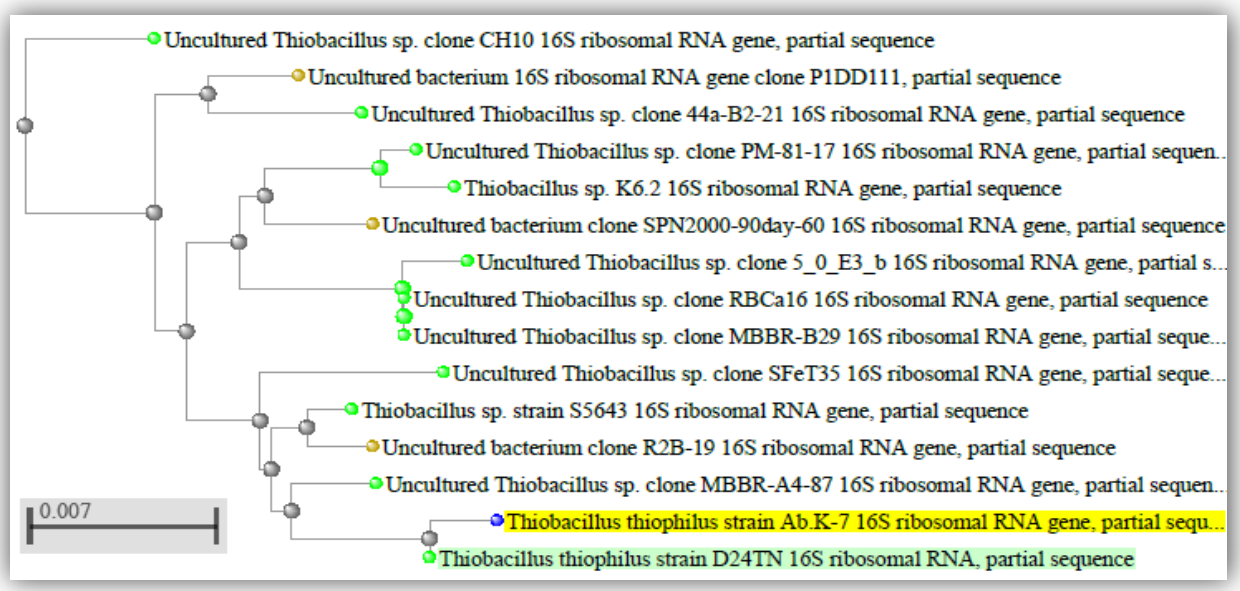


Figure (8:) Genetic tree of Thiobacillus thiophilus strain Ab.K-7

Table (9): Molecular diagnosis of bacterial isolates based on the percentage of identity of the 16S rRNA gene sequences with bacterial strains registered in the World Gene Bank at the NCBI website

Code of *Thiobacillus* isolates	Type and isolation of bacteria registered in the Global Gene Bank	Simila % rty	Country	Accession number	Highest match bacteria	Bacterial isolates
ON844981.1	*Thiobacillus thioparus* strain Ab.K-5	99.26	USA	NR_044755.1	*Thiobacillus thioparus strain Starkey*	B1
ON844984.1	*Thiobacillus denitrificans* strain Ab.K-8	99.26	The Netherland s	DQ407816.1	*Thiobacillus denitrificans strain DSM 739*	B2
ON844983.1	*Thiobacillus denitrificans* strain Ab.K-6	99.23	Germany	NR_025358.1	*Thiobacillus denitrificans strain NCIMB 9548*	B3
ON844982.1	*Thiobacillus thiophilus* strain Ab.K-7	99.17	Germany	NR_044555.1	*Thiobacillus thiophilus strain D24TN*	B4

CONCLUSIONS

Relying on the ITS region of the 16S rRNA gene was efficient in diagnosing the study isolates and gave a similarity of 9.17-9.26% with international isolates. We also conclude frome the resalts of the study that : all isolates of sulfur-oxidizing bacteria isolated from different sources and identified phenotypically and molecularly have the ability and efficiency to phosphate Solubilizing, produce IAA, chelating compounds, sulfates, and lower the pH, which in turn works to encourage plant growth by improving the chemical and fertility characteristics of the soil and providing nutrients to the plant, especially in the soil of arid and semi-arid areas, it can be used in the future to manufacture biofertilizer that enhances plant growth in sustainable agriculture and is environmentally friendly.

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