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ARTICLE

Biochemical and Molecular Identification of *Azospirillum brasilense* Bacteria and Evaluation of Their Efficiency in Producing Hormones, Dissolving Phosphorus, and Fixing Nitrogen

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ABSTRACT

The study aimed to isolate *A.brasilense* bacteria from the soil of the rhizosphere of different plants and different locations in Al-Diwaniyah Governorate. They were identified in two ways. The first was the routine method, which included studying the microscopic and cultural characteristics and biochemical tests of the isolates. The second method was molecular, using polymerase chain reaction (PCR) technology and using primers. It also included testing the efficiency of these isolates in dissolving tricalcium phosphate (TCP) on Pikovskaya agar medium, fixing nitrogen in the liquid nutrient medium (N.B), and measuring the amount of hormone production using an HPLC device. The results of isolation and regular and molecular identification the presence of ten isolates of bacteria bearing the characteristics of *A. brasilense* bacteria, out of fifteen local bacterial isolates, took the following symbols and sequences (Az2, Az3, Az5, Az6, Az7, Az9, Az11, Az12, Az13, Az14), as the results showed confirmation of the identification of the bacterial isolates identified by biochemical tests. Using a specialized primer to amplify the 462bp fragment of the 16S ribosomal RNA gene, the results of testing the efficiency of the bacteria in dissolving phosphate (TCP) showed that the isolate (Az13) outperformed the highest value in its effectiveness in dissolving metallic phosphorus through the diameter of the clear zone around the colony, which was effective in dissolving phosphate of up to 3.89 mm. As for the nitrogen fixation efficiency test, the isolate (Az3)

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for Auxins, Gibberellins, and Cytokinins, the isolate (Az3) recorded the highest value in the amount of Auxins production (Indol-3-acetic acid) its concentration reached 34.4 $\mu g.mL^{-1}$, and as for the production of the hormone Gibberellins (GA3), the isolate (Az3) recorded the highest value amounting to 34.7 $\mu g.mL^{-1}$, as for the production of the hormone Cytokinins (CK), The isolate (Az11) recorded the highest value, amounting to 28.8 $\mu g.mL^{-1}$.

Keywords: *A. brasilense*; PCR; Dissolving tricalcium phosphate Auxins; Gibberellins; Bacterial isolates

1. Introduction

Previously, biochemical identification was mainly used to isolate and identify microbes. Despite the remarkable and important developments in microbiology, a revolution occurred in biology and the appears of that called molecular biology, which studies biology at the molecular level. The emergence of molecular biology led to a significant increase in speed, sensitivity and the specificity of microbiological identification, and one of the most important techniques used in this field is the PCR (polymerase chain reaction) technique, which has become an important part of modern identification studies at the molecular level. PCR technology is an extracellular technology used to copy or amplify a specific sequence of DNA enzymatically and in a specific time, as it allows a small piece of DNA to be amplified millions of times. Sequential analysis of 16S rRNA has also led to the redefinition of the classification of many bacterial species [1,2]. Microorganisms play an important role in agricultural systems, especially Plant Growth Promoting Rhizobacteria (PGPR), as they contribute to plant growth through three mechanisms. They work firstly as biofertilizers, such as nitrogen-fixing bacteria and phosphate-dissolving bacteria; Which contribute to helping the plant obtain nutrients [3,4] and secondly asphytostimulators, which include microorganisms that produce phytohormones, such as the genus *Azospirillum* bacteria. *Azospirillum* bacteria are a type of free-living, nitrogen-fixing bacteria and have been used as a vital vaccine since the beginning of their discovery, their use in the field of biovaccines has increased on a large scale in the agricultural field recently due to their beneficial effects in improving plant growth indicators, which are not limited only to fixing atmospheric nitrogen, but are linked to the ability of bacteria to secrete compounds that encourage plant growth, which include the production of effective hormones, as they are among the most effective soil nutrients. Microflora produces hormones, including auxins, cytokinins, and gibberellins, which greatly affect root growth, which leads to improved absorption of water and nutrients, as well as the production of indole acetic acid, ethylene, salicylic acid, nitric oxide, and iron chelating compounds Sidrophores, and their ability to dissolve phosphate compounds [5,6]. Plant growth-promoting bacteria (PGPBs) of the genus Azospirillum have been the subject of extensive research in recent years. Apart from fixing nitrogen from the atmosphere, inoculating plants with Azospirillum also gives them phytohormones like indole-3-acetic acid, which are vital for plant growth. Some research indicate that Azospirillum, possibly through the release of phytohormones, is essential for providing plant tolerance to biotic and abiotic stressors. Azospirillum can increase plant growth under abiotic stresses through a variety of mechanisms, including antioxidants, osmotic adjustment, phytohormone production, and defense strategies like pathogen-related gene expression. This is due to an increase in phytohormone levels within the jasmonic acid/ethylene pathway. A thorough investigation of the processes that Azospirillum activates might make it feasible to employ PGPB as a key tactic to lessen the effects of biotic and abiotic stresses on agricultural productivity [7].

The objective of this study was to isolate and identify Azospirillumbrasilense bacteria from the rhizosphere soil of various plants in different locations within Al-Diwaniyah Governorate. The bacteria were identified using two methods: traditional methods involving microscopic, cultural, and biochemical analysis, and molecular methods employing polymerase chain reaction (PCR) technology with specific primers.

The study also aimed to assess the efficiency of these bacterial isolates in:

1) Dissolving tricalcium phosphate (TCP) on Pikovskaya agar medium. 2) Fixing nitrogen in a liquid nutrient medium. 3) Producing growth hormones, such as auxins, gibberellins, and cytokinins, using High-Performance Liquid Chromatography (HPLC).

2. Materials and methods

2.1 Collecting soil samples

Fifteen soil samples were collected from the rhizosphere (the area surrounding the roots) of plants and from various locations in Al-Diwaniyah Governorate, as shown in **Table 1**. These samples were used for the isolation and identification of *A. brasilense* bacteria. The plants were uprooted along with the roots and the surrounding soil after making a circle around the plant to accommodate its roots. The plants were carefully uprooted to ensure that the soil remained completely attached to the roots. The samples were then placed in polyethylene plastic bags that had been previously sterilized with alcohol, and the information for each sample was documented.

2.2 Isolation of *A. brasilense* bacteria

A.brasilense bacteria were isolated from the rhizosphere soil of different plants and different locations using the decimal dilution and plate counting method. 1 mL of the 10^{-6} dilution was added to test tubes containing nitrogen-free medium (Nfb), and the tubes were incubated at 30 °C for 72 h after appeared the white ring growth in the Nfb medium or its turbidity appeared several millimeters from the surface, which indicated the presence of growth of *Azospirillum* bacteria. Three successive transfers were made on the same medium and incubated at 30 °C for 48 h, then purified by transferring the visible growth into the Nfb medium using the carrier. (Loop) on plates containing R.C medium ((Red Congo) to which Congo red dye was added and cultured using the striping method. The plates were incubated at 37 °C for 72 h, and after the appearance of crimson-coloured colonies on the medium, each colony was purified again by re-striping it on the same medium to ensure its purity (**Figure 1**) According to the method[8, 9], bacterial isolates were identified based on microscopic and cultural characteristics and biochemical tests as previously reported[10], by studying their microscopic characteristics (cell shape, Gram stain, movement)[11], and studying their cultural characteristics (growth in the presence of Salt (3%) NaCL, growth at PH = 7.5–6, growth at temperatures 4–42, growth in Simmon's Citrate medium (according to[12], and studying its biochemical characteristics (Catalase test, Oxidase test, Gelatin liquefaction test, Urease enzyme production, methyl red test, test for the ability to produce indole, starch hydrolysis, test for the need for Biotin, test for the need for Pectin), for the purpose of confirming the identification of bacterial isolates belonging to the type *A. brasilense*, polymerase chain reaction (PCR) technology was used, as genomic DNA samples of the bacterial that numbered ten out of fifteen isolates were extracted by using a genomic DNA extraction kit for Gram-negative bacteria produced by FAVORGEN/Korea, and the primer was prepared according to the manufacturer's instructions. The primers preparation: The primers were lyophilized, they dissolved in the free ddH2O to give a final concentration of 100 pmol/µL as stock solution and keep a stock at −20 to prepare 10 pmol/µL concentration as work primer suspended, 10 µL of the stock solution in 90 µl of the free ddH2O water to reach a final volume 100 µL.

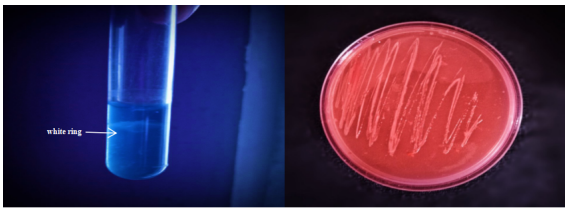

Figure 1. *A. brasilense* bacteria growing and forming a crust under the surface on (Nfb) medium and then purified on (R.C.) medium.

2.3 Testing the efficiency of the isolates in dissolving phosphate

Pikovskaya medium was inoculated, and the dishes were incubated at a temperature of 30 °C for a period of 5 to 7 days. Phosphate-dissolving bacterial colonies were identified by the formation of a clear, transparent halo around their colonies, as shown in **Figure 2**, which indicates the dissolution of phosphate. The equation described by[13] was used to express the bacteria's ability to dissolve phosphate.

Dissolution index (IS) = colony diameter + aura diameter/colony diameter.

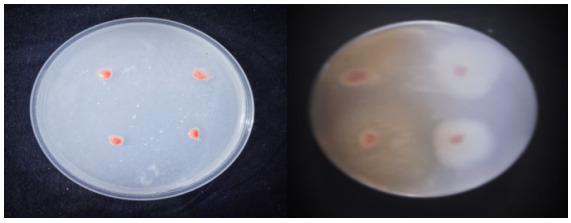

Figure 2. Formation of a clear, transparent halo around colonies on Pikovskaya agar medium.

Table 1. Isolate numbers, the source of the isolate, and the areas from which it was collected.

No	Symbol of *Azospirillum* isolates	Source of isolation	Region of isolation
1	Az1	Clover plant	al-Furat quarter
2	Az2	Barley plant	Al-Ahmed
3	Az3	Sweet bean plant	Al-Ahmed
4	Az4	Shallot plant	Ghamas
5	Az5	Clover plant	Al-Shamiyah
6	Az6	Wheat plant	Al-Shamiyah
7	Az7	Barley plant	Al-Daghara
8	Az8	Clover plant	Al-Daghara
9	Az9	Okra plant	Afak
10	Az10	Red pepper plant	Afak
11	Az11	Clover plant	Afak
12	Az12	Barley plant	Summer
13	Az13	Wheat plant	Summer
14	Az14	Barley plant	Al-Daghara
15	Az15	Clover plant	Al-Daghara

Table 2. The sequence of primer that used this study.

Primer	Sequence	Primer sequence 5'– 3'	Size of Product (bp)
Azospirillum	F	GGTAATAC-GAAGGGGGCGAG	462
16S ribosomal RNA gene	R	AGGGTTGGTAAG-GTTCTGCG	Primer design

2.4 Testing the efficiency of the isolates on fixing atmospheric nitrogen

Nitrogen-free liquid environments (Nfb) were prepared for the bacteria *A. brasilense*. 50 mL of the liquid environment was placed in 250 mL bottles and (1%) of a solution was added to each one. The bottles were inoculated by adding 1 mL of liquid culture for the different isolates and incubated with the shaking incubator (shaker) for 3 weeks at a temperature of 28 °C. The amount of ammonia formed in the environment was estimated by taking 2 mL of it and estimating it with a device (Microkildahl) **Figure 3**, which is explained by [14].

2.5 Extraction of hormones produced by *A. brasilense* bacteria

Grow strains of bacteria *A. brasilens* in medium (Nfb) supplemented with 100 µg.mL^{-1} of DL-Tryptophan. The medium is distributed into 250 mL bottles containing 100 mL of it, and the bottles are inoculated with 5 mL of the *A. brasilens* vaccine according to the method of Baron and, then incubated in a shaking incubator (100 rpm^{-1}) at a temperature of 28 °C for 24 h, and the bacterial cultures growing in 100 mL of the medium are extracted by centrifuging themg × 7700 in a centrifuge for 30 min according, then 50 mL of the filtrate (culture medium) was taken and the pH was adjusted to 2.5 as in Scheme No. (1) using 2N hydrochloric acid (HCl). After that, the partitioning process was performed with a similar volume of ethyl acetate by using a 250 mL separating funnel four times, then collecting the (organic phase) containing the plant hormones referred to above. The evaporation process was carried out using a rotary evaporator at a temperature of 35 °C according to the method of [15–17], then 5 mL of methanol alcohol was added to it, then the samples were analyzed using a high-performance (pressure) liquid chromatography (HPLC) device equipped by the Japanese company Shimadzu, type LC-6 A, equipped with a variable

Table 3. Biological materials & chemicals.

No.	Biological materials & chemicals	Company/Country
1	FavorPrep Total DNA Mini Kit	FAVORGEN/Korea
2	Master Mix or GoTaq® Green Master Mix	Promega/USA
3	TAE buffer10 X	Carl Roth/Germany
4	Red safe	Mebep Bio Science/China
5	6X Loading dye	Intron/Korea
6	Agarose	Carl Roth/Germany
7	Ladder 100	Transgen/China
8	Primer	Macrogen/Korea

Table 4. Reaction components of PCR.

Component	25 µL (Final volume)
Component	25 µL (Final volume)
Masret mix	12.5 µL
Forward primer	10 picomols/µL (1 µL)
Reverse primer	10 picomols/µL (1 µL)
DNA	1.5 µL

wavelength spectrometer (Spd). 6A-UV Spectrophotometer, where 20 microliter samples were thrown into the column using a Rheodgne-7120 injector at a temperature of 40 °C regulated by a Sil-6A thermal controller A C-18 reverse-phase column with dimensions of 250 × 4–6 mm-10 was used in the analysis, the (mobile phase) consists of phosphoric acid and methanol at a ratio of 40:60 v/v. Readings were then taken for each of the Auxins, Gibberellins and Cytokinins according to[18, 19].

3. Results and discussion

3.1 Results of microscopic and cultural biochemical diagnosis of bacterial isolates

Table 6 shows the fifteen local isolates belonging to the genus *Azospirillum* spp., which were isolated from the soil of the roots (Rhizosphere) of different plants and from different areas of Al-Diwaniyah Governorate, and were identified based on cultural and microscopic characteristics and biochemical tests. The isolates were characterized by slightly curved bacillary, vibrioid, or spiral shapes. It is short, and has a spiral movement in the liquid medium (similar to the movement of piercing a cork auger). It is also distinguished by its ability to fix nitrogen in the semi-solid, nitrogen-free culture medium (Nfb), forming a thin, white membranous growth (Pellicle) 1–1.5 cm below the surface of the culture medium, and upon continuing incubation at a temperature of 30 °C for 48 h, the membranous growth increased to 2–3 mm below the surface, meaning that it fixes nitrogen under conditions of little ventilation (microaerophilic). This characteristic is one of the distinctive signs of the genus *Azospirillum* bacteria, the color of the culture medium (Nfb) containing the bromothymol blue dye changed from light green at pH = 6.8 to basic blue due to the formation of ammonia upon nitrogen fixation. All isolates were negative for gram stain and positive for the oxidase and catalase enzyme examination, some bacterial colonies were dyed red and others light pink when grown in solid medium (R.C) containing Congo red dye, *Azospirillum* bacteria absorb this dye, so they appear different from other nitrogen-fixing bacterial species in terms of color. From the above, and based on what was reported in many previous studies, these characteristics belong to the genus *Azospirillum*, this was confirmed[20]. The results presented in **Table 6** also showed that all isolates were able to reduce nitrate and were unable to reverse nitrification, some isolates were also able to grow in the presence of NaCl at a concentration of 3%. Some isolates were also able to grow at pH 7.5 and 6.0, meaning they tolerate salinity and pH = 7.5. Some isolates also showed a negative result for the Biotin need test,

Table 5. The optimum condition of detection Lin0454.

No.	Phase	Tm (°C)	Time	No. of cycle
1	Initial Denaturation	95 °C	3 min	1 Cycle
2	Denaturation-2	92 °C	45 s	30 Cycle
3	Annealing	66 °C	45 s	
4	Extension-1	72 °C	45 s	
5	Extension-2	72 °C	7 min	1 Cycle

meaning growth of the isolates occurred in the presence and absence of Biotin, some isolates also showed the ability to decompose pectin within seven days, and all isolates were negative for the Indol test and unable to dissolve gelatin. Also, all isolates were positive for the MethylRed test and the Nitrate test, and when comparing these characteristics with the characteristics of species belonging to the genus *Azospirillum*, it can also be concluded from the differential characteristics between the different isolates belonging to the genus *Azospirillum* through the results of the phenomenon in **Table 6** that the following bacterial isolates (Az2, Az3, Az5, Az6, Az7, Az9, Az11, Az12, Az13, Az15) belong to the type of bacteria *A. brasilense*, these results are consistent with [21], and it is also clear from the results that the following isolates (Az1, Az8, Az10, Az15) belong to the *A. lipoferum* type of bacteria, as for the isolate (Az4), it belongs to the type of bacteria *A. irakense*, which is unique in its ability to use pectin as the sole source of carbon. It resists a certain concentration of salinity up to 3% and grows in media with a pH of more than 7.5. The reason for this difference between species of the same genus is because each of them possesses the characteristics of that species, and this is consistent with what was found by [22].

3.2 Molecular identification of A. brasilense isolates

Molecular diagnosis is one of the important and sensitive indicators and methods that confirm the microscopic and biochemical diagnosis of bacteria.It is considered one of the highly efficient taxonomic methods in determining the phylogenetic origin of bacterial species [23]. The primers (16SPSEfluR, 16SPSEfluF) were used to amplify the 16S ribosomal RNA gene using polymerase chain reaction (PCR) technology. The results showed that ten isolates were *A. brasilense* bacteria out of fifteen isolates that were tested biochemically, microscopically, and culturally, and the results were confirmed molecularly with the primer used, taking the following sequences and symbols (Az2, Az3, Az5, Az6, Az7, Az9, Az11, Az12, Az13, Az15). The ten strains showed clear bands with a molecular size of 462bp, a visible base pair (4). This is the expected size produced by this pair of primers with the DNA of the bacterium *A. brasilense*, which bound to its complementary sequences on the template DNA chain and did not bind to others, which was reflected in the results of electrophoresis of the amplification products using an agarose gel, which confirmed that all of these samples were *A. brasilense* bacteria. The DNA extracted from the bacterial isolates (Az2, Az9) was then sent to the South Korean company (The company is Microgen/Korea) to conduct a Sconesink analysis using the Sanker method to ensure the match rate The results showed a match rate between the ten isolates **Table 7** that ranged from 81% to 86%, and the match rate for the two isolates (Az3 and Az13) was 86%, which is the highest percentage among the bacterial isolates of the type *A. brasilense*, this is consistent with what was found [24] when using the above primer to diagnose *A.brasilense*.

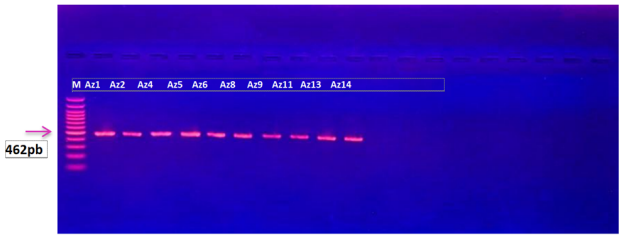

Figure 3. Gel electrophoresis for extraction of genomic DNA from bacterial samples on 1.5% acrose gel.

3.3 Efficiency of *A. brasilense* isolates in dissolving phosphate and fixing nitrogen.

The results of **Table 8** showed the ability of the isolates of bacteria *A. brasilense*. belonging to the genus *Azospiril-*

Table 6. Some cultural, microscopic and biochemical characteristics for diagnosing bacterial isolates.

Type of test	No. of isolates														
	Az1	Az2	Az3	Az4	Az5	Az6	Az7	Az8	Az9	Az10	Az11	Az12	Az13	Az14	Az15
Gram stain	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cell shape	Red Vibrioid	Red Vibrioid	Red Vibrioid	Red Vibrioid	Red Vibrioid	Red Vibrioid	Red Vibrioid	Red Vibrioid	Red Vibrioid	Red Vibrioid	Red Vibrioid	Red Vibrioid	Red Vibrioid	Red Vibrioid	Red Vibrioid
Colony color															
Denitrification	±	-	-	±	-	-	-	±	-	±	-	-	-	-	±
Nitrate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pectin	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
MethylRed	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Indol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Citrat	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Biotin	+	-	-	+	-	-	-	+	-	+	-	-	-	-	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Urease	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Starch	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gelatin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3% NaCl	±	+	+	+	+	+	+	±	+	±	+	+	+	+	±
pH=6	±	+	+	±	+	+	+	±	+	±	+	+	+	+	±
pH=7.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+ there's growth, - there's no growth, ± weak growth

lum on phosphate solubility and growth in Pikovskaya agar environment containing insoluble tricalcium phosphate, the results also showed that there is variation in the ability of bacterial isolates to dissolve solid phosphate, which proves that these bacteria have the ability to dissolve phosphate. The isolate (Az13) belonging to *A. brasilense* gave the highest effectiveness in dissolving phosphate, as the value of the phosphate dissolution coefficient reached 3.56 mm, it was followed by the isolate (Az11) belonging to *A. brasilense*, with a dissolution coefficient of 2.89 mm, and the lowest value of the dissolution coefficient was for the isolate (Az14). The value of the phosphate dissolution coefficient reached 1.79 mm, and this can also be explained by the ability of bacterial isolates of the *A. brasilense* type to produce phosphate-dissolving organic acids, this is consistent with what was found[25] that there are many bacterial species belonging to the genus *Azospirillum* that have the ability to dissolve phosphate. There are many studies that have indicated the role of different types in dissolving fixed phosphorus (Hafsa et al., 2019; Ikhajiagbe et al., 2020). As for the results of nitrogen fixation efficiency for bacterial isolates identified as *A. brasilense* shown in **Table 8**, the results showed the ability of bacterial isolates to fix nitrogen at different rates after (3) weeks of cultivation on nitrogen-free liquid medium (Nfb) at a temperature of 30 °C, the results showed that the bacterial isolates (Az3, Az13) belonging to *A. brasilense* had the highest efficiency in the amount of nitrogen fixation, superior to the other isolates, as they reached (12.44, 11.31) $mg.L^{-1}$, respectively. The lowest amount of nitrogen fixed was 6.60 $mg.L^{-1}$ when isolated (Az5). This is due to the role of enzymatic secretions, hormones and organic acids produced by bacteria, the role of which was reflected positively in increasing the amount of fixed nitrogen. These results are consistent with findings[26–31]. On the basis of the activity of the bacteria in dissolving phosphate and the amount of fixed nitrogen, the isolate (Az3, Az13) was chosen from the Bacteria isolates. *A. brasilense* as a bacterial vaccine.

3.4 Efficiency of *A. brasilense* isolates in producing hormones

The results shown in **Table 9** showed that there is a difference between the isolates of *A. brasilense* in the amount

Table 7. Results of diagnosing bacterial isolates using polymerase chain reaction (PCR) technology. 16S ribosomal RNA gene.

NO.	Sequence ID with compare	Source	Identities
Az2	ID: CP032348.1	Azospirillum brasilense strain MTCC4039 plasmid p4	82%
Az3	ID: CP032348.1	Azospirillum brasilense strain MTCC4039 plasmid p4	86%
Az5	ID: CP032348.1	Azospirillum brasilense strain MTCC4039 plasmid p4	85%
Az6	ID: CP032348.1	Azospirillum brasilense strain MTCC4039 plasmid p4	83%
Az7	ID: CP032348.1	Azospirillum brasilense strain MTCC4039 plasmid p4	85%
Az9	ID: CP032348.1	Azospirillum brasilense strain MTCC4039 plasmid p4	83%
Az11	ID: CP032348.1	Azospirillum brasilense strain MTCC4039 plasmid p4	84%
Az12	ID: CP032348.1	Azospirillum brasilense strain MTCC4039 plasmid p4	81%
Az13	ID: CP032348.1	Azospirillum brasilense strain MTCC4039 plasmid p4	86%
Az15	ID: CP032348.1	Azospirillum brasilense strain MTCC4039 plasmid p4	83%

of hormone production using the (HPLC) device (**Figure 4**), and the isolate (Az3) of *A. brasilense* recorded the highest value in quantity. Production of auxins (indole-3-acetic acid) at a concentration of 34.4 $\mu g.mL^{-1}$, respectively. The lowest amount of indole-3 acetic acid production was recorded in the isolate (Az14) at a concentration of 20.5 $\mu g.mL^{-1}$. As for the production of the gibberellins hormone, the bacterial isolate (Az3) recorded the highest amount of gibberellins. The isolate (Az12) belonging to the same type of bacteria recorded the lowest amount of gibberellins, amounting to 34.7 $\mu g.mL^{-1}$. It reached 18.5 $\mu g.mL^{-1}$. As for the production of cytokinins, the isolate (Az11) of the A. brasilense plant recorded the highest amount of cytokinins production at a concentration of 28.8 $\mu g.mL^{-1}$. The lowest concentration of cytokinins produced by the isolate (Az9) of the same type of bacteria was 8.19 $\mu g.mL^{-1}$. This difference and difference between isolates may be due to the nature and ability of E bacteria to produce these growth regulators, and the nitrogen-fixing bacteria *A. brasilense* isolated from the roots of different weeds differ in their production of growth regulators among the same isolates. Classify. This depends on the concentration of tryptophan added to the medium, and many researchers have indicated that the age of the bacterial culture affects the production of hormones until it reaches a

Table 8. Efficiency of *A. brasilense* isolates in dissolving phosphate and fixing nitrogen.

Symbol of isolates	Amount of solvated phosphate			Amount of fixed N2 mg·L^{-1}
	Colony diameter mm	Zone of halo Mm	SI	
Az2	3.5	5.8	2.66	8.68
Az3	2.9	5.2	2.79	12.44
Az5	3.7	6.6	2.78	6.6
Az6	4	8.4	3.1	9.68
Az7	4.6	8.2	2.78	10.12
Az9	2.9	6.6	3.28	8.16
Az11	3.8	7.2	2.89	10.41
Az12	4.9	10.6	3.16	7.5
Az13	3.2	8.2	3.56	11.31
Az14	3.4	2.5	1.74	7.12

steady state[32–43].

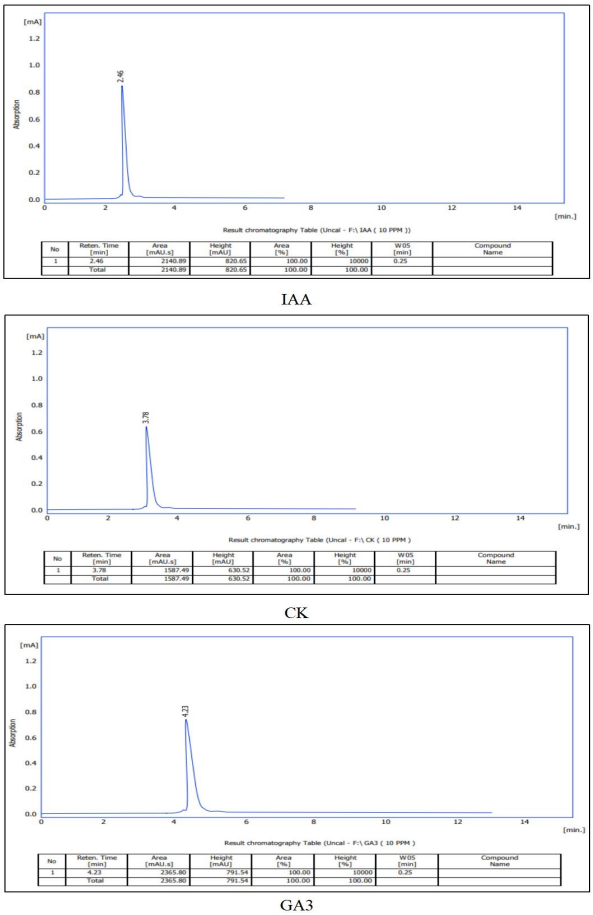

Figure 4. Estimation of hormones using (HPLC) device.

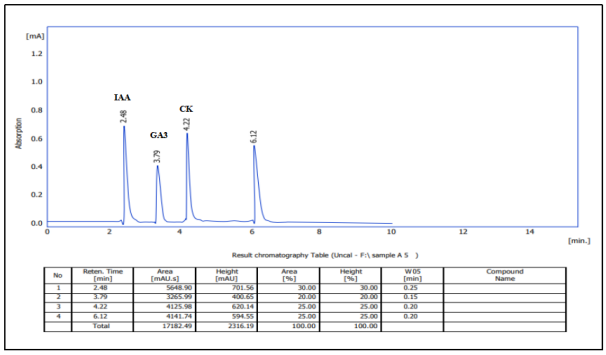

Figure 5. Estimating a hormone IAA,CK and GA3 using (HPLC) a device.

4. Conclusion

The study successfully isolated and identified ten strains of *A. brasilense* from the rhizosphere soil in Al-Diwaniyah Governorate using both routine and molecular methods. The isolates demonstrated various efficiencies, with Az13 excelling in dissolving tricalcium phosphate, and Az3 showing superior nitrogen fixation and hormone production capabilities. These findings highlight the potential of *A. brasilense* isolates in enhancing soil fertility through phosphate solubilization, nitrogen fixation, and hormone production. these bacte ria have the ability to dissolve phosphate. The isolate (Az13) belonging to *A. brasilense* gave the highest effectiveness in dissolving phosphate, as the value of the phosphate dissolution coefficient reached 3.56 mm.

Table 9. Quantity of IAA, GA3, and Ck resulting from isolates of *A. brasilense* bacteria.

Symbol of isolates	IAA	GA3	Ck
Az2	27.8	19.8	23.4
Az3	34.4	34.7	23.3]
Az5	28.2	25.7	28.0
Az6	28.9	25.4	24.6
Az7	29.8	28.7	24.1
Az9	21.9	21.6	19.8
Az11	24.8	34.3	28.8
Az12	22.0	18.5	25.5
Az13	32.0	27.2	25.3
Az14	20.5	18.6	23.2

Author Contributions

All authors contributed equally to all stages of the study, from conceptualization and study design to data collection, analysis, writing of the manuscript, and final approval of the published version.

Conflict of Interest

The Authors declares that there is no conflict of interest.

Data Availability Statement

Data will be available on request from the author.

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