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Isolation and Evaluation of Local Exopolysaccharide (EPS)-Producing Bacteria from Drought-Affected Soil: Insights into Stress Tolerance and Antioxidant Capacity

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Abstract

Drought and Stress are one of the most important environmental factors that affect the effectiveness of natural microbes in the soil. Twenty local bacterial strains were obtained from thirty soils experiencing drought. The isolates were identified morphologically and microscopically into 10 isolates as *Azotobacter* spp. and 10 are *Bacillus* spp. All isolates were tested for the ability to produce exopolysaccharide EPS. It was found that every isolate was productive, with EPS yields ranging from 1.19 to 1.95 for *Bacillus* spp. and 1.14 to 2.25 for *Azotobacter* spp. The isolates that produced the most EPS, Ba1, Ba5, and Ba7 from *Bacillus* spp., and Aoz4 and Aoz8 from *Azotobacter* spp., were selected and subjected to five different doses of polyethyleneglycol (PEG-6000) (0%, 10%, 15%, 20%, 25%) to test their ability to withstand drought. Ba1 and Ba7 significantly outperformed and were more tolerant than the Ba5 isolate and the most EPS-producing isolates Aoz4 and Aoz8 from *Azotobacter* spp. were selected and exposed to drought tolerance by exposing them to five successive concentrations of polyethyleneglycol (PEG-600) (0%, 10%, 15%, 20%, 25%). Biological and chemical tests were performed on the five isolates selected in the study, and the following were diagnosed as 3 isolates of *Bacillus subtillis*, while the other two were *Azotobacter chroococcum*. The isolates showed resistance to induced drought when exposed to double concentrations of (PEG-6000) with stable production of EPS. EPS appeared to have antioxidant activity in the extract, reaching 79% at a concentration of 200 µg/mL, with an IC50 of 11.735 µg/mL.

Keywords: *Azotobacter chroococcum, Bacillus subtillis*, antioxidant activity, glycol Polyethylene (PEG-6000), Drought, exopolysaccharide.

Introduction

Prolonged moisture deficiency impairs plant growth, and its negative impact is further exacerbated when combined with heat stress and drought[1,2] . Drought is a recurring disturbance in the water cycle, significantly disrupting terrestrial carbon cycles. It also limits plant growth and is associated with increased risk of wildfires, the spread of invasive species, and habitat loss with accelerating climate change, drought

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frequency, and severity are increasing globally[3,4,5]. Complex and dynamic interactions between microbes, roots, soil, and water in the rhizosphere accountable for modulate the physical, chemical, and structural properties of soil[6,7]. Microbial sugars help bind soil particles together, helping to form small and large aggregates that enhance structural stability. However, drought stress can lead to reduced microbial activity and agricultural productivity, a growing challenge in the face of climate change[7]. These bacteria improve root growth and increase their efficiency in absorbing micronutrients[8,9,10]. In turn, the roots release a range of organic compounds into the soil in the form of exudates, which are a rich source of carbon, attracting various microbes, including PGPR[11,12].These bacteria contribute to regulating the water balance of plants in rainfed agriculture by promoting root system growth[13]. They also improve water use efficiency (WUE) and increase the roots' ability to absorb water during drought[14]. Studies have proven their effectiveness in improving crop growth and yield in both greenhouses and agricultural fields[15,16]. These positive effects of PGPR are attributed to several mechanisms: Some strains produce diverse compounds, such as plant metabolites (e.g., hydrogen cyanide (HCN) and 2, 4-diacetylphloroglucinol (DAPG), antibiotics (e.g., phenazines), and growth-promoting volatile compounds[16,17]. Other strains produce exopolysaccharides, siderophores, biofilms, and phytohormones, which in turn influence plant physiological processes[18].

Microorganisms secrete physiologically active exopolysaccharides (EPSs), extracellular polymers that contribute to soil moisture conservation[19]. Most of these EPSs are heterogeneous polymers with an average molecular weight of approximately 8 x 10^4 Daltons and contain multiple monosaccharides such as arabinose and xylose, and contain hydroxyl, phosphate, carboxyl, N-acetyl, amine, methyl and sulfate groups Under drought conditions, higher glucose, proline, and free amino acid contents were observed in plants inoculated with EPS-producing bacteria[20.21] .Furthermore, inoculation of Zea mays with different strains of Pseudomonas spp. contributed to improved soil stability around the roots and increased the formation of soil aggregates, leading to improved aeration and soil water retention[21].

EPS contributes to the permanent attachment and colonization of microorganisms to roots by forming a fibrous network that firmly attaches bacteria to the root surface. These EPS are produced by soil bacteria in the form of sticky, capsular substances that can be absorbed by clay surfaces to form a protective layer around soil aggregates[22]. Thanks to EPS-producing bacteria, plants are better able to adapt to drought conditions. Plants treated with EPS-producing Azospirillum bacteria showed greater drought tolerance, likely due to improved soil structure and increased soil cohesion. Increased root tissue growth was also observed in sunflower plants infected with an EPS-producing bacterial strain under drought stress[21,23]. However, maize seedlings inoculated with Bacillus spp. exhibited physiological responses indicating mitigation of the negative effects of drought. These included increased plant biomass, higher relative water content, improved leaf moisture, increased root-adhering soil/root tissue ratio, improved soil aggregate stability, and reduced leaf water loss[21,24].

Inoculation also reduced the activity of antioxidant enzymes (ascorbate, peroxidase, catalase, and glutathione peroxidase), while proline content increased under drought condition[25]. As a result, plant biomass improved due to lower levels of reactive oxygen species and increased antioxidant enzyme activity when inoculated with plant growth-promoting bacteria compared to the control group under an irrigation regime equivalent to 80% of field capacity. It is worth noting that many microorganisms, such as Bacillus

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spp. and Pseudomonas spp., are known to produce EPS, but their identification and characterization still require further study and expansion[26].

Therefore, the current study using environmentally friendly methods and the appropriate time, and preferably through growth-promoting bacteria (PGPB) that produce drought-tolerant and exopolysaccharides and the subsequent antioxidant capacity of these EPS, specifically to understand their role in protecting against additional stresses and their potential use in applications that combat soil drought.

Material and Methods

Sample Collection

A total of 30 soil samples were collected from different areas of the Babylon Governorate. These samples included sandy desert soil. The samples were collected according to the method described by Aslim *et al.* [٢٧]. A small shovel was used to clean the soil and excavate it to a depth of 15 cm from the ground surface. The samples were placed in clean, sterile tubes and transferred to the laboratory.

Bacterial isolation

Soil samples were cleaned from plant residues, and each gram of sample was suspended in 99 ml of sterile distilled water and shaken vigorously for 2 minutes. The samples were heated in a water bath at 80°C for 30 minutes, then serial dilutions (10^{-1} – 10^{-6}) were made from the liquid. After cooling to room temperature. 0.1 ml of each dilution was withdrawn and spread using L-shaped glass diffuser in a Petri dish containing nutrient medium (two replicates were performed for each dilution). The samples were incubated at 37°C for 48 hours to establish bacterial colonies[٢٧].

Purification of isolates

The purification of isolates was carried out by transferring a portion of the growing colony individually using a sterile loop carrier to new Petri dishes containing nutrient medium. The cultures were spread using a streaking method, and the process was repeated several times to obtain pure colonies.

Isolation of *Bacillus* spp.

Media used

The prepared media were sterilized in an autoclave at 121°C for 15 minutes. agar Nutrient, Nutrient broth were used to isolate, purify, and preserve *Bacillus* bacteria [٢٨].

Initial diagnosis of *Bacillus* spp.

Microscopic examinations are primarily used to identify and diagnose *Bacillus* spp bacteria. These examinations rely on observing the various characteristics of *Bacillus* spp under a microscope, including characteristics such as shape, size, cell arrangement, and staining characteristics, and Gram staining of

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bacteria. The spores were examined using the Fulton-Schaeffer method, where malachite green stain was introduced into the spores by evaporating the bacterial emulsion[٢٩].

Isolation of *Azotobacter* spp.:

Soil samples were carefully placed inside sterile polyethylene bags, and the soil was mixed well ١mm of soil was placed in sterile test tubes containing 9 ml of normal saline solution (1-1 Solution), thus obtaining a dilution of 10^{-1}. A series of decimal dilutions was performed. 0.1 ml was taken from each dilution and spread in a tube containing solid SMS (Salt Mineral Sucrose) medium, where it worked for 96 hours in an aerobic environment. Two replicates were used for each dilution and incubated in an incubator at 37°C.

The prepared media were sterilized in an autoclave at 121°C for 15 minutes.

Sucrose Mineral Salts broth media

This medium was prepared according to the method of Abbasniayzare *et al*[٣٠] by adding 10 mM of sucrose and 3 mM of calcium carbonate to 100 ml of a solution consisting of 0.5 mM of potassium phosphate monohydrate (4HPO2K), 0.2 mM of aqueous magnesium sulfate (MgSO4. $7H_2O$), 0.1 mM of calcium sulfate (CaSO4.$7H_2O$), and 0.02 mM of ferrous sulfate (FeSO4. $7H_2O$). 0.02 mM of potassium sulfate, 0.01 mM of hydrated manganese iodide ($MnSO_4$), and 0.01 mM of potassium molybdenum oxide (MoO3) were added to the medium. Two % agar-agar was added to the medium. After the medium was prepared, the pH was adjusted to 7.2 and the volume was added to one liter of distilled water. This was then sterilized in an autoclave and poured into a sterile container. This medium was used to estimate the total number of Azotobacter bacteria.

Sucrose Mineral Salts agar medium

This medium was used to grow Azotobacter bacteria. It was prepared in the same manner as described by Abbasniayzare *et al*[٣٠], with the addition of 1.5% agar-agar to the medium. After the reconstitution process was completed, it was sterilized in an autoclave and poured into sterile Petri dishes.

Motility Test Medium

This medium was prepared by adding 4-5 mm of agar-agar powder to 13 mm of nutrient-broth powder. The contents were dissolved in distilled water. After completion of the reconstitution, the volume was increased to 1 liter. The medium was distributed into tubes and sterilized using an autoclave. This medium was used to detect bacterial motility[٣١].

Initial diagnosis of *Azotobacter* spp.

Azotobacter isolates were identified according to Holt and Krieg[32]. Identification was based on morphological and microscopic characteristics. Colony morphology on solid SMS medium, including shape, color, and size, was recorded. Selected colonies were examined microscopically using Gram staining

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to determine cell shape and staining properties. Motility was assessed by stab inoculation in motility medium followed by incubation at 37°C for 24–48 h. Growth spreading beyond the stab line indicated bacterial motility.

Quantitative screening of exopolysaccharide production from *Bacillus* spp.

After screening, all of the isolates used in this investigation were first determined to be Bacillus species. Luria broth medium was utilized. Following the manufacturer's instructions, this medium was produced, divided into five milliliter tubes, and autoclave-sterilized. Using a sterile loop, bacterial colonies from isolates grown on the nutritional medium—each one separately—were transferred to the tube containing Luria broth medium to create the vaccine. To obtain the vaccine, the tubes were incubated for 24 hours at 37°C[٣٤].

Preparation of the production medium

I used the Mineral Growth Medium, which consists of the following ingredients dissolved in a liter of distilled water: 5 mm yeast extract, 5 mm magnesium sulfate (MgSO4.7), 3 mm aluminum sulfate, and 50 mm glucose. This medium was prepared from the ingredients mentioned above. The ingredients were weighed, then added to a quantity of distilled water, and the pH was adjusted to 5 then, the volume was completed to 1 liter, and the medium was distributed in 75 ml glass flasks, with 19.6 ml in each flask and three replicates for each isolate [٣٥].

Inoculation of the production medium

At a wavelength of 600 nm, the production medium was infected with an inoculum volume equal to 2% of the medium volume and an optical density of 0.5 according to the McFarland standard. For 48 hours, flasks were incubated at 37°C with a shaking speed of 100 rpm in a shaking incubator [٣٥].

Extraction of exopolysaccharides from *Bacillus* spp.

Exopolysaccharide extraction was done using the Kanmani et al[٣٦] technique. The culture media was kept in a water bath at 90°C for 10 minutes following the 48-hour incubation period. A centrifuge set to 8000 rpm for 10 minutes was used to extract the cells from the fermentation medium. While the filtrate was added to 8% v/v trichloroacetic acid (TCA) and allowed to sit at 4°C for three hours, the cells were dried in order to determine the dry weight of the biomass. The protein in the medium was precipitated by centrifugation at 8,000 rpm for 10 minutes. The filtrate was then combined with two volumes of 95% cold ethanol and stored at 4°C for a full day. The precipitate was removed .Centrifugation was used to separate the EPS for 12 minutes at 12,000 rpm. To determine the dry weight, the precipitate was dried for 24 hours at 40°C while the filtrate was disposed of. The ten isolates with the highest productivity were chosen.

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Quantitative screening for exopolysaccharide production from *Azotobacter* spp. isolates

Inoculum preparation

Use liquid SMS medium to prepare the vaccine. Dispense the medium into 5 ml tubes, sterilized in an autoclave. Prepare the vaccine by transferring an *Azotobacter* isolate using a full loop from the isolate grown on solid SMS medium to the designated tube containing the liquid SMS medium prepared above. Incubate the tubes in an incubator at 37°C for 18 hours to obtain the vaccine [٣٧].

Preparation of the production medium

Broth Ashby nitrogen-free medium was used, which consists of the following materials:(K2SO4, 0.1%), (NaCl, 0.2%), (MgSO4·7H2O, 0.2%), (KH2PO4, 0.2%), (mannose 20%), and (CaCO3.5%). The aforementioned materials were weighed and mixed in a quantity of distilled water. After completion of the reaction, the volume was adjusted to 1 liter, after adjusting the pH to 7.0 [٣٨]. The medium was distributed into 2-liter glass flasks, with 49.5 ml in each flask, with three replicates for each isolate, and sterilized in an autoclave at 121°C for 15 minutes[٣٩,40].

Inoculation of the production medium

A specific gravity of 0.5 and an inoculum volume equal to 1% of the medium volume were used to inoculate the production medium at a wavelength of 540 nm. The medium was then incubated for 72 hours at 30°C with a shaking speed of 150 rpm/cycle[39,41].

Testing the ability of the isolates under study to tolerate drought conditions:

Evaluation of Drought Tolerance Potential

Drought tolerance testing was performed as follows:

1. To assess the ability of bacterial isolates to multiply under drought stress, different concentrations of polyethylene glycol (PEG 600) were added to the nutritional broth (NB) medium at different drought potential levels.
2. The water potential of NB medium was adjusted by adding 600 PEG at several concentrations (0%, 10%, 15%, 20%, and 25% (w/v)). For a whole day, 1% of the cultivated bacterial isolates were added to these solutions[42].
3. A photometer was used to measure the OD density at a wavelength of 600 nm following a 24-hour incubation period at 30°C in a shaking incubator running at 200 rpm.
4. The bacterial culture's development was examined at various PEG concentrations, and the relative growth rates (in relation to the PEG-free control) were computed[43].

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5. Exopolysacharide-producing bacterial isolates were selected for each bacterial species (*Azotobacter* spp., and *Bacillus* spp.) for their tolerance to drought conditions. The isolates were then evaluated for their ability to extract sugars and study their antioxidant properties[44].

Antioxidant Activity Test :DPPH Radical Scavenging Activity Test

To evaluate the antioxidant activity of EPS extract. The extract scavenging activity assay by DPPH (1-diphenyl-2-picrylhydrazyl) was performed. Two milliliters of extract with the following quantities were added: 200, 150, 100, 50, 25, 12.5, 6.25, and 3.125 g/ml. After half hour, absorbance was estimated at 517 nm.The extract was tested three times at every concentration. The formula below was used to compute the percentage reduction of DPPH (Q).

$$Q = 100 \times (A0 - AC) / A0$$

Where: Q (% reduction)

A0 (Absorbance of control sample without the antioxidant compound)

AC (absorbance of the test sample containing the antioxidant compound)

Absorbance of the two samples after 30 min incubation .

The IC50 value means the concentration of extract by which 50% of DPPH particles were scavenged. It was calculated from the plotted graph of scavenging activity against the various concentrations of the extract [45].

Results and Discussion

Isolation and identification of Bacillus spp.

In this study, 30 soil samples were collected from various drought-affected areas of the Babylon Governorate. Pure isolates of *Bacillus* spp were obtained from all collected samples, which had phenotypic characteristics identical to those of the bacteria to be isolated when using Nutrient medium for growth. Table 1 shows the cultural and microscopic characteristics of 10 *Bacillus* spp isolated, which were given the names Ba1-Ba10.

Table 1. The cultural and microscopic characteristics of *Bacillus* spp. isolates

Isolate	Form of Colonies	Gram Stain	Cell Form	Oxygen Requirement
Ba1	Dry, white colonies with irregular margins	+	*Bacillus*	Aerobic

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Ba2	Dry, brown colonies with irregular edges	+	*Bacillus*	Aerobic
Ba3	Dry, white colonies with irregular margins	+	*Bacillus*	Aerobic
Ba4	Dry, white colonies with irregular margins	+	*Bacillus*	Aerobic
Ba5	Wet, brown colonies with irregular edges	+	*Bacillus*	Aerobic
Ba6	Dry, brown colonies with irregular edges	+	*Bacillus*	Aerobic
Ba7	Dry, brown colonies with irregular edges	+	*Bacillus*	Aerobic
Ba8	Wet, brown colonies with irregular edges	+	*Bacillus*	Aerobic
Ba9	Dry, white colonies with irregular margins	+	*Bacillus*	Aerobic
Ba10	Dry, brown colonies with irregular edges	+	*Bacillus*	Aerobic

Isolation and identification of *Azotobacter* spp.

A total of 10 isolates were collected from soil samples collected from different areas of the holy Babylon Governorate and were cultured on SMS (salt mineral sucrose) medium, which is the appropriate medium for the growth of this type of bacteria. Table 2 shows the cultural and microscopic characteristics of 10 *Azotobacter* spp. isolated, coded Az1-Az10.

Table 2. The cultural and microscopic characteristics of *Azotobacter* spp. isolates

***Azotobacter* spp.**	**Form of colonies**	**Gram stain**	**Cell Form**	**Cyst**	**Oxygen requirement**
Azo 1	Smooth, shiny colonies, orange color.	-	Bacillus	+	Aerobic
Az0 2	Smooth, shiny colonies produce a mucous layer and white color	–	Bacillus	+	Aerobic

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Azo 3	Smooth, shiny colonies produce a mucous layer and a white color	-	Bacillus	+	Aerobic
Azo 4	Smooth, shiny colonies produce a mucous layer and orange. color	-	Spherical	+	Aerobic
Azo 5	Smooth, shiny colonies, orange color	-	Spherical	+	Aerobic
Azo 6	Smooth, shiny colonies produce a mucous layer and are orange. color	-	Bacillus	+	Aerobic
Azo 7	Smooth, shiny colonies produce a mucous layer and a white color	-	Spherical	+	Aerobic
Azo 8	Smooth, shiny colonies, white color	-	Spherical	+	Aerobic
Azo 9	Smooth, shiny colonies, white color	-	Spherical	+	Aerobic
Azo 10	Smooth, shiny colonies produce a mucous layer and white color	-	Spherical	+	Aerobic

Screening of *Bacillus spp* isolates for extracellular polysaccharide (EPS) production

The capacity of each isolated *Bacillus* species to create EPS was tested, and the most effective isolates were identified by measuring the dry weight of EPS.

The EPS-producing isolates, EPS dry weight, and cell dry weight are displayed in Table 3. By evaluating the ratio (dry weight of EPS / dry weight of cells), the most effective one for EPS generation was identified. The three *Bacillus* spp. bacterial isolates with the greatest ratio were chosen.

Table 3. Screening of *Bacillus* spp. isolates for extracellular polysaccharide (EPS) production

Isolates	Dry weight of EPS mg/L	Dry weight of cells mg/L	Dry weight of EPS/Dry weight of cells
Ba1	816	420	1.94
Ba2	912	500	1.82
Ba3	618	461	1.34
Ba4	906	700	1.29
Ba5	733	415	1.91

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Ba6	600	400	1.5
Ba7	456	234	1.95
Ba8	456	398	1.19
Ba9	765	564	1.35
Ba10	589	399	1.47

Screening of *Azotobacter* spp. isolates for extracellular polysaccharide production

All ***Azotobacter*** spp isolates obtained were subjected to testing for their ability to produce EPS, and the most efficient ones were determined by calculating the dry weight of EPS. Table 4 shows the isolates that produce EPS, the dry weight of EPS, and the dry weight of cells. The most efficient of them for EPS production was determined by calculating the ratio (dry weight of EPS / dry weight of cells). Two isolates of *Azotobacter* spp. bacteria with the highest ratio were selected.

Table 4. Screening of *Azotobacter* spp. isolates for extracellular polysaccharide (EPS) production

Isolates	Dry weight of EPS mg/L	Dry weight of cells mg/L	EPS/Cell Dry Weight Ratio
Azo1	120	100	1.2
Azo2	240	200	1.4
Azo3	618	461	1.34
Azo4	720	320	2.25
Azo5	467	399	1.17
Azo6	600	500	1.2
Azo7	956	834	1.14
Azo8	660	320	2.06
Azo9	765	564	1.35
Azo10	270	190	1.42

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Estimating the ability of *Bacillus* spp. isolates to withstand drought conditions.

Ba1, Ba5, and Ba7 were chosen from the ten isolates that produced the greatest EPS, respectively, and exposed to five different PEG concentrations (0%, 10%, 15%, 20%, and 25%) to assess their capacity and effectiveness in withstanding drought. The results indicated that isolation showed that the Ba5 strain was much outperformed and less tolerant than the Ba1 and Ba7 isolates. According to Table 5, the growth density value attained (0.730) and (0.730) OD, respectively.

Table (5): Drought-resistant efficiency of *Bacillus* spp. isolates under different concentrations of polyethylene glycol (PEG).

Isolate	0% PEG	10% PEG	15% PEG	20% PEG	25% PEG	Average
Ba1	0.930	0.800	0.720	0.650	0.550	0.730
Ba5	0.520	0.410	0.390	0.320	0.290	0.386
Ba7	0.950	0.820	0.770	0.640	0.560	0.730

L.S.D (0.05):

- Between PEG treatments = **0.054**
- Between isolates = **0.024**

Estimation of the tolerance of *Azotobacter* spp. isolates to drought conditions

According to Table 6, the isolates (Azo4) and (Azo8) were more tolerant than the other isolates when exposed to five successive doses of PEG (0%, 10%, 15%, 20%, and 25%). Their OD values were 0.582 and 0.586, respectively.

Table 6: Desiccation resistance efficiency of *Azotobacter* spp. isolates under different concentrations of polyethylene glycol (PEG).

Isolate	0% PEG	10% PEG	15% PEG	20% PEG	25% PEG	Average
Azo 4	0.730	0.530	0.390	0.370	0.320	0.468
Azo 8	0.710	0.580	0.510	0.450	0.390	0.528

L.S.D (0.05): Between PEG treatments = **0.058**; Between isolates = **0.026**

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Screening of isolates for exopolysacchride (EPS) production after exposure to withstand drought conditions.

After exposing the GPE-600 isolates to testing for their ability to produce EPS, the results were found to be very similar to those obtained in Tables 3 and 4. This indicates the ability of bacteria to tolerate drought and the role of EPS in protecting microbes from harsh environmental conditions [46].

Biochemical identification of the most drought-tolerant EPS-producing isolates

When conducting biochemical tests on the *Bacillus* isolates obtained in this study, it became clear that all isolates were positive for the gram stain and were motile, and they were also negative for the oxidase and urease tests, as shown in Table 7. Two duplicates of the bacteria were cultivated on solid nutritional medium. The ability to produce indole is shown in Table 7.

Table 7. Morphological and biochemical tests of *Bacillus* spp. (Ba1, Ba5, Ba7) and *Azotobacter* spp. (Azo4, Azo8)

Test	*Bacillus* spp. (Ba1, Ba5, Ba7)	*Azotobacter* spp. (Azo4, Azo8)
Catalase test	+	+
Oxidase test	–	+
Urease test	–	+
Indole test	–	+
Methyl Red	–	–
Kovac's	+	–
Citrate test	+	+
Movement (Motility)	+	+
Growth in 1% NaCl and glycerol	NoR	+
Growth in 0.1% phenol	NoR	+
Gelatin hydrolysis	+	+
Growth at 4–42°C	NoR	NoR

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Lactose utilization	+	NoR
H_2S production	NoR	+
Glucose utilization	+	+

Legend: + = Positive result; – = Negative result; **NoR** = No result

Five bacterial isolates were identified as the most productive of EPS and the most drought-tolerant. Four isolates (Ba1, Ba5 & Ba7) were identified using the Vitek2 device, and two isolates were identified using biochemical tests (Aoz4, Aoz8). Based on the results of microscopic examination and biochemical tests, and referring to the Berges factory[47] . the isolates were identified as shown in Table 8.

Table 8: Diagnosis of bacterial isolates

Isolate	Diagnosis	VITEK 2 Identification
Ba1	*Bacillus subtilis* 1	99%
Ba5	*Bacillus subtilis* 2	97%
Ba7	*Bacillus subtilis* 3	93%
Azo4	*Azotobacter chroococcum* 1	According to Table 7
Azo8	*Azotobacter chroococcum* 1	—

DPPH Scavenging Activity Assay

The 2,2-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity test was used to assess the antioxidant activity of the EPS extract. The results in Table 9 showed that the extract's dose-dependent antioxidant activity reached 79% at a concentration of 200 µg/ml, with an IC50 of 11.735 µg/mL, as illustrated in Figure 1.

Table 9: Percentage of DPPH scavenging activity (antioxidant activity) at various concentrations of ESP

No.	Concentration (µg/mL)	Absorbance (Ab.)	Scavenging Activity (%)
1	0	0.85	0
2	3.125	0.43	49
3	6.25	0.37	56

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4	12.5	0.31	64
5	25	0.27	68
6	50	0.24	72
7	100	0.22	74
8	200	0.18	79

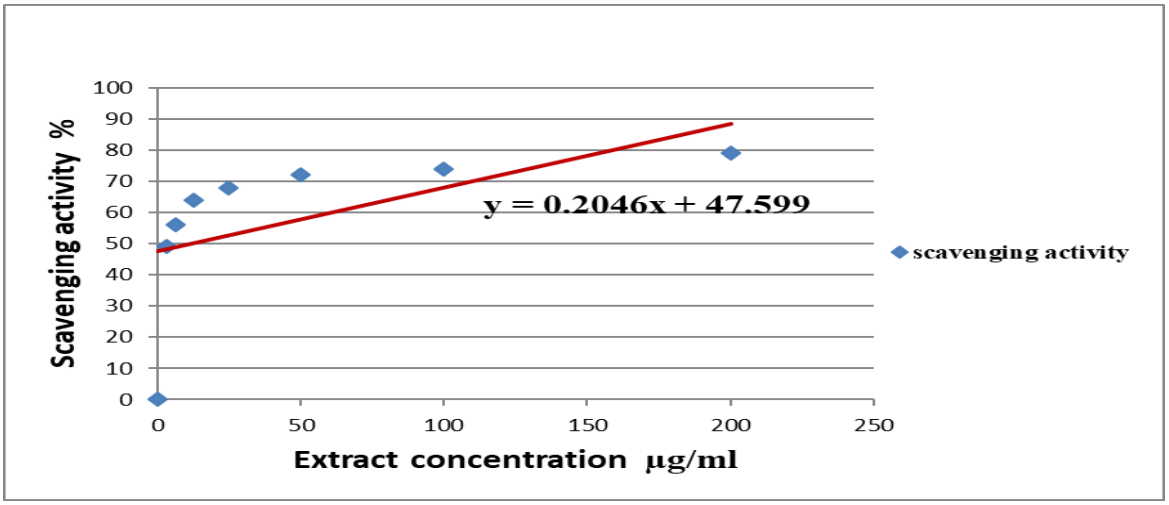

Figure (1): Dose response curve of EPS extract on DPPH free radical scavenging activity.

These findings align with those of Mohammed *et al.*[48] and Elkholy *et al.* [49]. To select potential bacilli for testing, colonies with advantageous growth characteristics can be chosen from a culture of an environmental sample that has been heavily diluted after heat shock or hot air drying[50]. Typically, cultured colonies are large, dispersed, and asymmetrical. Under a microscope, Bacilli cells appear rod-like and many are swollen due to oval endospores at one end[51]. On solid isolation medium (SMS), Azotobacter bacteria appeared as glossy, smooth colonies, some secreting a mucus coating. They varied in size and color from white to orange. The bacteria had a spherical shape and exhibited various morphologies, from spherical to rod-shaped, as noted in Saribay [52]. They could also form cysts. The stain tests negative for the gram stain. They depending on the type, and are found singly, in pairs, or in regular groups, sometimes as Holt & Krieg[32] mention. Tables 3 and 4 show that all isolates produced EPS, consistent with studies by Hindersah et al.[53], Bhandary & Alagesan [54], and Ranjbar et al.[55] . EPS production depends on factors such as the culture medium's composition, incubation time, temperature, and pH. Generally, EPS production increases when the medium is rich in carbohydrates and nitrogen [54]. It reaches its maximum after 72 hours of incubation and gradually increases as the pH deviates from 7, peaking at a pH of 7-8 [56]. Isolates showed variability in EPS production under the same conditions, including temperature, pH, nutrient type, and incubation periods. Differences among isolates depend on the strain type within the same bacteria [57]. While variation

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within a strain relies on genetic factors and the genes that encode EPS production, a study by Argianas [58] indicated that differences in EPS production for the same strain persist even after deleting the genes responsible for EPS synthesis[58]. Heavy metals, such as mercury, can influence EPS production. Some Azotobacter spp. isolates can grow and produce EPS in the presence of mercury, while others cannot. These differences are crucial for understanding the roles of specific exopolysaccharides in biofilm formation, heavy metal tolerance, and plant growth promotion[53]. Under various environmental stresses, the growth of extracellular glucose synthesis was enhanced[59]. Ghosh et al. [46] found that as drought-stress bacteria increased, so did the amount of EPS produced[59,46]. Tsegaye et al.[60] suggested that bacterial strains that produce extracellular polysaccharides (EPS) can survive under different stress conditions, while Collins and Margesin [58]proposed that microorganisms produce EPS as a stress response to protect their cells from adverse external circumstances. Spore production, a feature of these bacteria, was observed during incubation[62]. If oxygen is available or utilized, cultivated Bacillus species produce spores and test positive for catalase[63]. All Azotobacter isolates from this investigation tested positive for catalase and oxidase in biochemical tests, consistent with the findings of Idowu et al.[64]. They could grow in 1% glycerol and NaCl, were motile, and showed no signs of gram staining, aligning with Sachin[47]. Although they could not grow in 0.1% phenol, high concentrations of phenol are toxic to some *Azotobacter* species, consistent with Revillas et al.[65]. Most phytochemical compounds have antioxidant mechanisms, including: (1) scavenging ROS; (2) upregulating or protecting antioxidant defenses; and (3) preventing ROS formation by inhibiting enzymes or chelating trace elements involved in free radical generation[46,48] . Conclusion

Drought-exposed soils have diverse microbial communities, with *Bacillus* spp. and *Azotobacter* spp. being the two dominant species in Babylon's dry soils. Both have a high capacity to produce exopolysaccharides. When exposed to additional stress, their EPS production remained unaffected, demonstrating stable production levels. Both species exhibit high tolerance and productivity, and their EPS also show increased antioxidant activity.

Conclusions

This study showed that drought-affected soils contain diverse microbial communities, with *Bacillus* spp. and *Azotobacter* spp. as the dominant species in the soils of Babylon. Both species showed high EPS production, which remained stable under additional stress conditions. In addition, both species demonstrated good resistance and productivity, and their exopolysaccharides exhibited increased antioxidant activity.

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Author's Declaration

- We hereby confirm that all the Tables in the manuscript are original and have been created by us.
- This approval underscores our commitment to ethical research practices and the well-being of our participants.

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