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ISOLATION, SCREENING, AND CHARACTERIZATION OF PLANT GROWTH PROMOTING AZOTOBACTER SPECIES FROM RHIZOSPHERIC SOIL

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ABSTRACT

Numerous environmental factors that adversely impact agricultural production, plant development and seed germination frequently limit plant growth and productivity. Azotobacter sp. and other beneficial soil microbes are known to be efficient Rhizobacteria that promote plant growth through a variety of ways. With an emphasis on their capacity to produce the phytohormone Indole-3-acetic acid (IAA). Rhizospheric soil samples were taken from three distinct locations, obtained a total of thirteen bacterial isolates. Four isolates that showed notable IAA production were chosen for additional analysis after a preliminary screening for IAA production was completed. The chosen isolates were then examined for a number of characteristics that promote plant growth, such as biological nitrogen fixation, ammonia production, siderophore production, phosphate solubilization, and the synthesis of extracellular enzymes like catalase, protease, amylase, and chitinase. The findings showed that the chosen isolates had significant IAA production in addition to a variety of plant growth-promoting characteristics, suggesting their potential use as biofertilizers for sustainable agricultural systems. These results demonstrate the significance of rhizobacteria that produce IAA in enhancing soil fertility and plant growth.

KEYWORDS: Azotobacter sp., Screening Test, and Extracellular Activity of Catalase, Protease, Amylase, and Chitinase Enzymes

1. INTRODUCTION

A significant and enduring change in the expected weather patterns of a region or the planet over a prolonged period is known as a climatic shift. It concerns aberrant fluctuations in the climate. It could take tens, hundreds, or even millions of years for these changes to occur. However, increased anthropogenic activity including industrialization, urbanization, deforestation, changes in land use patterns, greenhouse gas emissions, temperature increases, variations in precipitation, and an increase in atmospheric CO_2 concentrations are all part of climate change scenarios. Two environmental factors impact agricultural yields i.e. biotic and abiotic stresses. Abiotic stressors cause plant morphological, physiological, biochemical, and molecular changes that negatively impact growth and output. It includes climatic stresses (heat, cold, flood, drought, etc.) and soil parameters (pH, physicochemical, and biological variables). Conversely, biotic factors encompass arthropods, diseases, weeds, vertebrate pests, anthropogenic evolution, and helpful creatures like pollinators, decomposers, etc. If not properly monitored and managed, several variables pose a serious risk to farms and reduce productivity. The three technical, biological, and environmental categories can be used to organize these factors [1].

Many nations are under pressure to produce more crops, which has led to an increase in the amount of land used for agriculture and intensification of cropland management techniques like irrigation and the heavy use of chemical fertilizers and synthetic pesticides for weed and disease control [2]. These methods have led to deterioration in the soil and water quality, accelerated soil erosion, contaminated groundwater, and decreased food quality. To overcome these climate changes, we have to use biofertilizers that help to increase soil fertility and propagate it by using different kinds of biofertilizers.

Biofertilizers are composed of living microorganisms like bacteria, fungi, or algae that are natural in origin and are utilized to boost soil quality and promote plant development. Biofertilizers fix nitrogen, solubilize minerals, and enhance plant growth directly or indirectly methods. There are several kinds of Biofertilizers like Nitrogen fixers biofertilizers (*Rhizobium, Azotobacter, Azospirillium,* are used), minerals like P, Zn, and K solubilizing biofertilizers (*Bacillus, Pseudomonas, Frateuria, Mycorrhizae,* are used), as well organic matter decomposers, etc. Advanced tools like *Azotobacter,* Blue-green algae, *Azospirillum, mycorrhizae,* and p-solubilizing microbes are being utilized as biofertilizers in agriculture, providing multiple benefits to the sector. Among this here focused on *Azotobacter* as a Nitrogen fixer biofertilizer [22].

Plant growth-promoting *Azotobacter* is increasingly used in agriculture because it provides a useful substitute for chemical fertilizers, insecticides, and other hazardous additions [3]. These *Azotobacter* bacteria create enormous amounts of growth-promoting chemicals that either directly or indirectly affect the general morphology and physiology of the crops. The utilization and diversity of *Azotobacter*, their capacity to colonize new areas, and the mechanisms of action that can be employed to support their application as a dependable component in the management of sustainable agricultural

systems are all necessary for the recent advancements in the field of sustainable development [4].

Azotobacter is a gram-negative, aerobic, free-living, oval, or round-shaped soil bacterium that creates cysts in unfavorable conditions [5]. Usually, they are polymorphic with sizes varying from 2 to 10 µm in length and 1 to 2 µm in width. In 1901, Dutch microbiologist *Beijerinck* and colleagues identified the genus *Azotobacter* as the initial nitrogen fixer that thrives aerobically and independently. These bacteria use atmospheric N_2 for their protein synthesis, which is then mineralized in the soil, providing crop plants with a significant amount of available nitrogen from the soil. *Azotobacter spp.* is vulnerable to low pH levels, elevated salt levels, and high temperatures [6]. They offer benefits to the growth and productivity of crops by creating biologically active compounds, promoting rhizosphere microbes, producing inhibitors for plant diseases, changing how nutrients are absorbed, and increasing nitrogen fixation in a biological way. There are 7 species in the genus Azotobacter, such as *Azotobacter croococcum, A. armeniacus, A. beijerinckii, A. paspali, A. salinestris, A. nigricans,* and *A. vinelandii*. They vary in size from 1-2 µm in width and 2-10 µm in length [7].

Azotobacter chroococcum in agriculture has demonstrated its significance in enhancing plant health and enhancing soil quality [8]. The use of *Azotobacter chroococcum* in research experiments as a microbial inoculant, by releasing growth substances and their effects on plants, has significantly enhanced crop production in agriculture. Various strains of Azotobacter have been discovered to have the ability to synthesize amino acids when cultured in media containing different sources of carbon and nitrogen [9]. The substances generated by these Azotobacter play a role in various processes that contribute to promoting plant growth [10]. This study looks at the threat caused by the use of chemical fertilizers and pesticides and the potential of Plant growth promoting Azotobacter as an eco-friendly and sustainable alternative.

2. MATERIALS AND METHODS

2.1 Sample collection:

Soil samples were obtained from the rhizosphere of agricultural plants in various areas of Madhupur village (21.0323° N, 70.6001° E) in Talala, Gir Somnath district in order to isolate possible PGPR (plant growth-promoting rhizobacteria) from these locations. Sample collection occurred at the time when crops were actively growing, ensuring that the samples were taken from metabolically active rhizosphere microbial populations. Plants were carefully uprooted and collected with soil on their roots using sterile collection instruments. Soil loosely attached to the root was removed by carefully shaking it off, and soil that was tightly adhered to the root was collected by brushing the root surface with a sterile spatula. Approximately 100 g of rhizosphere soil was collected from each sample site. Soil samples were collected from the root zone (5 – 15 cm deep) of sampled plants and placed into sterile plastic bags. The sterilized bag that contained each soil sample was labeled.

2.2 Sample processing:

Weighed 10 grams of rhizospheric soil, which was aseptically added to 100 ml of sterile 0.8% n-saline to prepare soil suspension. For one hour, soil suspensions were shaken at 200 RPM for an hour. After that, sterile distilled water was used to perform a series of decimal dilutions up to 10^{-2} in test tubes.

2.3 Isolation:

Soil samples were initially inoculated on Nutrient Agar for the isolation of bacterial colonies. The obtained isolates were subsequently transferred onto Ashby's Mannitol Agar (AMA) medium by four flame method and incubated for 24-48 hours at 30º C to confirm their nitrogen-fixing ability. In this media, only those isolates can grow which are nitrogen fixers in nature. For the purity of the cultured isolates, the bacterial culture process was done three times [11]. A morphological and biochemical test was used to identify the isolates.

2.4 Characterization of Isolates:

2.4.1 Colonial characterization

Colony characterization of all the isolates was done by physical observation of colony size, shape, margin, elevation, opacity, pigmentation, and appearance according to experimental microbiology by Cappuccino, J.G. and Sherman, N.

2.4.2 Morphological characterization

According to Experimental Microbiology by Cappuccino, J.G. and Sherman N. Gram staining and capsule staining techniques were performed for the microscopic characterization of all isolates.

2.4.3 Biochemical characterization

All isolates were chemically characterized with IMViC tests – Indole production test, Methyl red test, Voges Proskauer test, and Citrate utilization test as mentioned in Experimental Microbiology by Cappuccino, J.G. and Sherman, N.

2.5 Screening of Isolates for Indole-3-acetic acid production:

The procedure described by Bric et al. was used to qualitatively screen bacterial isolates for the synthesis of indole-3-acetic acid (IAA). After inoculating 10 mL of nutritional broth with 0.1% L-tryptophan, the isolates were cultured for 48 hours at 30 ± 2 °C. The negative control was uninoculated broth with 0.1% L-tryptophan. One millilitre of the culture was centrifuged for ten minutes at 10,000 rpm following incubation. After mixing the supernatant with an equivalent volume of Salkowski reagent, it was left in the dark for half an hour. Positive IAA synthesis by the isolates can be observed by the development of a pink to red colouring.

2.6 Plant Growth Promoting Traits:

2.6.1 Ammonia production

A bacterial culture grown overnight was inoculated with 10 ml of peptone broth and incubated for 48 hours in a shaking position at 30°C. Following incubation, 0.5 ml of Nessler's reagent was added. The brown to yellow color indicates a positive ammonia production test [16].

2.6.2 Siderophore production

The generation of siderophores was measured in isolates using the Chrome azurol S agar medium. After preparing and dividing Chrome Azur S agar plates into equal sectors, test organisms were spot inoculated, and the plates were then incubated at 30^0C for 48–72 hours. The formation of a yellow-to-orange halo zone surrounding the growth was considered favourable for synthesizing siderophores [17].

2.6.3 Phosphate solubilisation

The plates were prepared with Pikovskaya's medium. The cultures were spotted on the plates and incubated at 30^0 C for 5 days. After incubation observing a clear zone of solubilization indicated a positive result a distinct halo zone close to the bacterial growth was measured and by using the following equation calculated the phosphate solubilization index [19].

$$\text{Phosphate solubilization index (SI)} = \frac{\text{Colony diameter} + \text{Halo zone diameter}}{\text{Colony diameter}}$$

2.6.4 Hydrogen Cyanide (HCN) Production

The method suggested by Castric was used to determine the amount of hydrogen cyanide (HCN) produced by selected isolates. On nutritional agar enriched with glycine (4.4 g/L), the isolates were streaked. The underside of the Petri dish lid was covered with a strip of Whatman filter paper soaked in a mixture of 0.5% picric acid and 2% sodium carbonate (Na_2CO_3). After sealing the plates with Parafilm, they were incubated for 48 to 72 hours at 30 ± 2 °C. Following incubation, the filter paper's colour changed from yellow to light brown, brown or reddish-brown indicating that the bacterial isolates were producing HCN.

2.7 Extracellular enzyme activity

2.7.1 Catalase activity

Place 1-2 drops of culture on a clean glass slide and add 1-2 drops of 30% H_2O_2. Formation of effervations indicates a positive result.

2.7.2 Protease Activity

Selected isolates were spotted on SMA–skimmed milk agar and incubated for 48 hrs at 30°C. The zone of utilization surrounding bacterial colonies was observed to assess the ability of the colony to produce protease [20].

2.7.3 Amylase Activity

Isolates were spotted on Starch agar plates and incubated for 48 hrs at 30°C. Gram's iodine was flooded on the incubated plate surface. A positive result is indicated by the formation of a clear halo zone around the colonies and the development of dark blue to purple-blue colour in the surrounding medium after the addition of Gram's Iodine. [21].

2.7.4 Chitinase Activity

The selective media was used to evaluate chitinase production. Media was contained g/L: colloidal chitin-1 %(W/V), Na_2HPO_4-6, K_2HPO_4-1, NH_4Cl-0.5, KH_2PO_4-3, $MgSO_4.7H_2O$-0.12, NaCl-0.5, yeast extract-0.05, agar-15 and set pH 7. Selected isolates were inoculated on this plate and incubated at 30°C for 4 days [18].

2.8 Abiotic stress tolerance of isolates

2.8.1 Salt Tolerance

By inoculating the culture suspension into Nutrient Broth (NB) supplemented with varying concentrations of Sodium chloride (1%, 3%, and 5%). Bacterial growth was observed based on turbidity of the broth, indicating tolerance to different salt concentrations. when the plates were incubated at 30 °C for 24 to 48 hours, which indicates tolerance to different salt concentrations.

2.8.2 pH Tolerance

Bacterial suspensions were inoculated into Nutrient Broth adjusted to various pH levels (5, 7, 9, and 11) in order to assess the isolates' pH tolerance. To determine a suitable and tolerable pH range, inoculated broths were incubated at 30 °C for 24 to 48 hours, and growth was recorded based on turbidity to determine the optimal and tolerable pH range.

2.8.3 Temperature Tolerance

By inoculating Nutrient Broth and incubating the cultures at various temperatures (20° C, 30° C, 40° C, and 45° C), the temperature tolerance of the bacterial isolates was evaluated. The turbidity of the broth, which indicates tolerance to the corresponding temperature conditions, was used to assess bacterial growth after 24 to 48 hours of incubation.

3. RESULTS AND DISCUSSION

3.1 Isolation, morphological and biochemical characterization of isolates:

13 rhizospheric bacterial isolates were obtained from soil samples in the Madhupur village of the Gir Somnath district. Microscopic features including Gram and capsule staining were used to assess their staining capacity observed under a 100X oil emulsion lens, and morphological features including colony characteristics, including size, shape, margin, elevation, appearance, pigment, opacity and the observations and biochemical analysis shown in Tables 1 and 2 were used to identify the isolates. All thirteen isolates were screened for Indole-3-acetic acid (IAA) production. Among them, four isolates showed positive results, indicated by the development of a pink to reddish coloration after the addition of Salkowski reagent. These positive isolates were MAND5, MCKD2, MDKD1, and MFK11 considered potential Plant Growth-Promoting Rhizobacteria and were selected for further characterization and evaluation. The methods for identification were derived from Bergey's Manual of Determinative Bacteriology (JF, M. 2000). This identification technique led to the designation of MDKD1 as *Azotobacter sp.*

3.2 PGP traits activities of isolates:

The present study characterizes direct mechanisms including plant growth-promoting traits (PGP traits) viz. Indole Acetic Acid (IAA) production, Ammonia production, Siderophore production, Solubilization of Phosphate, Extracellular enzyme activity of catalase, protease, amylase, and chitinase enzyme.

3.2.1 Indole-3-Acetic Acid (IAA) Production:

IAA production was quantified with the use of Salkowski's reagent by spectrophotometric method. Color development

was visible at the highest IAA concentration within a minute and continued to increase in dark conditions for 30 minutes. Therefore, the optical density was measured after 30 minutes. The intensity of the dark reddish pink color development in the tube checked spectrophotometrically at 530 nm indicated the strong IAA synthesis by the selected isolates. IAA production in nutrient broth, with 0.1% L-tryptophan ranged from 29.67 to 148.27 µg/ mL. Amongst all four isolates, IAA production was higher in the isolate MDKD1 and lower in the isolate MAND5. Table 3 describes the data of IAA production and Figure 1 shows the color changes in test tubes.

3.2.2 NH_3 Production:

NH_3 production was spectrophotometric quantified by the Nessler's reagent at 450nm. Change in colour development from yellow to brown or orange suggested the highest NH_3 concentration within 2-3 minutes. All isolates produced ammonia in varying concentrations of 19.44 to 48.83 µg/mL (Table 3 and Figure 2).

3.2.3 Siderophore Production:

Siderophore production was observed by the yellow or orange halo zone formation around the colony on the CAS agar medium. From selected four isolates two isolates were produced siderophore. Table 3 describe the data of siderophore production.

3.2.4 Phosphate Solubilization:

The phosphate solubilization was estimated quantitatively using Pikovskaya's agar as described by Mahdi et al., 2013. The efficacies of phosphate solubilization are shown in Table 3. When the zone of solubilization was detected surrounding the isolated colony on Pikovskaya's agar, then the solubilization index (SI) was calculated with the equation mentioned in the protocol using the colony's diameter and zone. Amongst the studied rhizospheric isolates, two isolates solubilized the phosphate MDKD1 and MFK11, as well two isolates did not solubilize phosphate – MAND5 and MCKD2.

Figure 1: IAA Production

Isolates	MAND5	MCKD2	MDKD1	MFK11
Size	Medium	Small	Medium	Medium
Shape	Irregular	Circular	Circular	Circular
Margin	Entire	Entire	Entire	Entire
Elevation	Raised	Convex	Raised	Flat
Opacity	Opaque	Opaque	Opaque	Translucent
Texture	Rough	Mucoid	Mucoid	Smooth
Appearance	Dull	Shiny	Slimy	Shiny
Pigment	Off white	Pale yellow	Cream	Cream
Gram reaction & shape	Gram Negative & short rod	Gram Positive & short rod	Gram Negative & short rod	Gram Negative & rod

Table 1: Colony characteristics of the isolated rhizobacteria

Isolates	Parameters										
	Indole test	Methyl red test	Voges Prausker test	Citrate utilization	Oxidase test	Catalase test	Glucose	Lactose	Sucrose	H_2S Production	Starch utilization test
MAND5	-	+	+	+	+	+	+	+	+	-	+
MCKD2	-	-	+	+	-	-	+	-	+	-	+
MDKD1	+	-	+	+	+	+	+	+	+	+	+
MFK11	-	-	+	+	+	+	+	-	-	+	+

Positive results (+) , Negative result (-)

Table 2: Biochemical characteristics of the isolated rhizobacteria

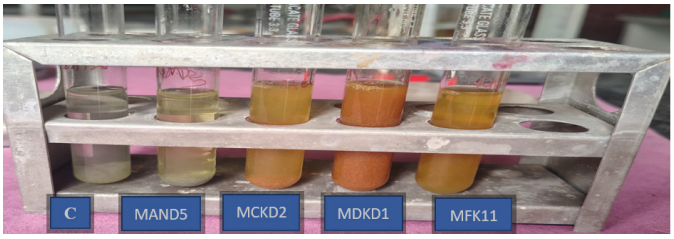

Figure 2: Ammonia Production

3.2.5 Extracellular enzyme activity:

Hydrolytic enzymes secreted by rhizospheric bacteria play a crucial role in breaking down different pathogen components. As shown in Table 3, the hydrolytic enzyme secretions were measured using a plate test. Out of all selected isolates, three isolates showed catalytic activity, two isolates showed proteolytic activity, all four isolates showed amylase activity and only one isolate showed chitinase activity.

3.3 Screening of isolates:

3.3.1 Salt Tolerance Assay:

All isolates were inoculating them with additional concentrations of NaCl salt at 1%, 3% and 5% in a sterile nutrient broth. MAND5 and MDKD11 are particular isolates that can withstand a 5% concentration of NaCl salt.

3.3.2 pH Tolerance Assay:

All of the selected isolates tolerate a pH range, including 5, 7, 9, and 11. pH 5 are intolerable to all isolates. No isolate was able to grow its pH below 7 and 11 or above. Therefore, every isolate was an alkaliphile.

3.3.3 Temperature Tolerance Assay:

A narrow temperature range, including RT, and 37°C, is tolerated by the selected isolates. Room temperature and 37° C temperature were tolerated by all the selected isolates. Half of the isolates could grow at 20° C and 45° C temperatures.

No.	Name	IAA Production. µg/mL	NH3 Production µg/mL	Siderophore Production	P. Solubilization Index (SI)	Catalase Production	Protease activity	Amylase activity	Chitinase activity
1	MAND5	29.67	-		-	+		+	+
2	MCKD2	31.55	19.44		-	-		++	-
3	MDKD1	148.27	48.83	+++	2.19	+	++	++	-
4	MFK11	42.53	23.56	++	1.79	+	+	+	-

Positive results indicate like +++ (Strong), ++ (Medium), + (Moderate), - (Negative result)

4. CONCLUSION

A total of 13 bacterial species were isolated in the current study from the soil sample collected in the village of Gir Somnath district. Out of the 13 isolates, 4 isolates (MAND5, MCKD2, MDKD and MFK11) have able to produce IAA, according to the data. The finest PGP features include the capacity to withstand a variety of pH ranges (5, 7, 9, and 11) and temperature ranges (20° C, 30° C, 40° C, and 45°C), as well as the ability to produce ammonia, Siderophore, solubilize phosphate, and exhibit catalase, protease, amylase and chitinase activity. Every test result is positive in the MDKD1 and MFK11 isolates. Thus, we believe that these particular species of bacteria can be used as biofertilizers.

5. FUTURE PROSPECT:

The future of this research on *Azotobacter spp.* focus on developing sustainable biofertilizers to enhance crop yield, reduce the use of chemical fertilizers, and improve soil health. It also explores genetic modification and microbial consortia for eco-friendly agriculture and climate change mitigation.

6. DATA AVAILABILITY STATEMENT:

The raw datasets supporting the conclusions of this article will be made available by the authors, without undue reservation. Raw data can be found in online repositories. The names of the repository/repositories are cited in the article.

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