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# Studies on Isolation of Plant Growth Promoting *Azotobacter spp* from Soil Samples

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ABSTRACT

Soil is the heterogeneous mixture of different kinds of microorganisms. Soil provides nutritive environment for the growth of microorganisms. One of the soil microbes is Azotobacter. The aim of present study was to isolate plant growth promoting Azotobacter spp. In the present study, 25 soil samples were collected from different locations of Akola and Akot region. In the study, 10 Azotobacter spp. were isolated. Azotobacter spp. was checked for phosphate solubilizing ability, stress tolerance (Temperature, pH, Salt), Nitrate Reduction, Cyst formation by using Burks medium and Indole Acetic Acid production using L- Tryptophan. Amongst the 10 isolates all were found to reduced nitrate, NH3 formation ability and IAA production, while two isolates H3 and C5 were only found to be have phosphate solubilizing ability. The seed germination test was also performed, Azotobacter showed promising results on the moth beans. The result reveals that Azotobacter could be a better source for crop improvement and soil fertility over the use of chemical fertilizers.

**Keywords:-** Azotobacter spp., Nitrate Reduction, Phosphate Solubilization, IAA.

## I. INTRODUCTION

The first representative of the genus, *Azotobacter chroococcum,* was discovered and described in 1901 by the Dutch microbiologist and botanist Martinus Beijernick. These species are found in neutral and alkaline soil (Gandora and Bhardwaj, 1998; Martyniuk and Martyniuk, 2003). Members of this genera, are mesophilic, which require optimum temperature of about 30°C (Gomare *et al.,* 2013). They are gram negative, aerobic, pleomorphic, motile, non-symbiotic, non-endospore former, live in chains, clumps or singly, may or may not provided by flagella and their resting stage is spent as a thick walled cyst, which protects the organism from harsh climate (Samal *et al.*, 2020). It includes four genera: *Azomonas, Beijernickia, Derxia and Azotobacter* (Shaikh and Shakir, 2018).

*Azotobacter vinelandii* and *Azotobacter chroococcum* are the most ubiquitous species found but other species includes *A. beijerinckii, A. paspali, A. agilos, A. insignis, A. macrocytogens, A. tropicalis.*

Researchers are more interested in *Azotobacter* because of its versatility is being applicative in many spheres.

Biofertilizers is a substance which contains living beneficial microorganisms, on applyimg to seeds, plant surfaces or soil, it promotes growth of plant by making availability of primary nutrients by solubilizing phosphorus, seiderophore secretion, fixing atmospheric nitrogen. *Azotobacter* also produces growth harmones such as riboflavin, gibberllins, auxins, cytokines, and Indole Acetic Acid.

Due to increased population in the world, there is need to increase the crop productivity by using various chemicals fertilizers, insecticides and pesticides. The soil and environment has been affected badly due to the tremendous use of the chemical fertilizers in agricultural field and hence the essential nutrients from the soil is diminishing. Moreover, the chemical fertilizers are very much expensive. In view of this background information, the present study was undertaken to isolate, identify the *Azotobacter species* with various plant growth promoting properties and it's effect on seed germination.

## II. MATERIAL AND METHODS

- **Collection of soil samples**

The 25 Soil samples were collected from different places of Akola and Akot region. The 10 gm of soil samples were collected from 15 cm with sterile spatula in a sterile polythene bags.

- **Isolation and Identification of *Azotobacter spp.***

1 gm of soil sample was inoculated into the Nitrogen Free Mannitol broth and incubated at room temperature for enrichment upto 3 days. After 3 days the broth was inoculated on sterilized Azotobacter agar medium plates and further incubated for 3 days at 28°C. The isolated colonies were streaked on the Azotobacter agar slants and incubated as pure culture. Isolates were characterized by using Gram staining, colony (shape, color, margin, size and texture). Biochemical test was also performed. Cyst formation by isolates on Burk's medium was performed. Isolates were stained by crystal violet and observed under oil immersion objective for cyst formation.

- **Screening of isolates for growth promoting characteristics**

**Indole Acetic Acid Production test**

The IAA production test was performed as per the protocol of Mankar and Barate (2018). Yeast Extract Mannitol broth with the addition of L – Tryptophan was inoculated by isolates and incubated at room temperature for 2 days. Non inoculated broth culture was kept as control. The 1 ml of supernatant was then mixed with 2 ml of Salkowski's reagent (1.5ml of 0.5 M $FeCl_3$, con. $H_2SO_4$, 50 ml D.W). The mixture was kept in dark for 45 minutes at room temperature. Red colour developed was recorded and calculated with spectrophotometer at 530 nm by using standard curve.

**Ammonia Production Test**

Bacterial isolates were inoculated in peptone water and incubated for 48 hours at 28°C. After incubation, Nessler's reagent (0.5 ml) was added to it. Formation of yellow colour shows positive test for Ammonia Production (Cappuccino and Sherman, 1992).

**Nitrate Reduction Test**

For nitrate reduction test, isolates were inoculated into nitrate broth and incubated at 28°C for 3 days. After incubation the sulphanillic acid and α-napthylamine mixture (1:1) is added. The development of pink colour shows the nitrate reduction test positive (Samal *et al.*, 2020).

**Phosphate Solubilization test**

Isolates were inoculated in Pikovskaya agar and incubated at 28°C for 2 – 5 days. The clear zone indicates solubilization of phosphate by isolates (Samal *et al.*, 2020).

- **Determination of stress tolerance by isolates**

**Temperature tolerance**

To find out the optimum temperature for growth, nitrogen free mannitol broth was inoculated and incubated at different temperatures (viz. 15°C, 28°C , 37°C, 40°C, 60°C). The growth was checked by taking OD at 620 nm.

**Salt tolerance**

Nitrogen free mannitol broth of different concentration of sodium chloride (viz. 2%, 4%, 6%, 8,%, 10%) were inoculated and incubated at 28°C for 48 hours. The growth was checked by taking OD at 620 nm.

**pH tolerance**

To find out the optimum pH for growth (viz. 5, 6, 7, 8, 9) nitrogen free mannitol broth was prepared, which was maintained by using diluted HCl and NaOH and inoculated 28°C for 48 hour. The growth was checked by taking OD at 620 nm.

- **Seed germination by roll towel method**

Bacterial isolates were grown in respective N - Free Mannitol broth at 28°C for 48 hours. Moth beans were sterilized and soaked by broth. Effect on seed germination by the isolates, using the roll towel method was studied. Soaked seeds by inoculant of each isolates were then sown in each pot. A control was taken by using distilled water for comparison. Root and shoot length of germinated plants were measured after four weeks.

## III. RESULTS

The present research work was carried out with an objective to study the characteristic features of *Azotobacter species.* The 25 soil samples were collected from Akola and Akot region. Out of total 25 soil samples, 10 isolates were obtained on Azotobacter Isolation agar. The isolates were subjected to morphological, cultural and biochemical test (Table 1). All the 10 isolates were gram negative and colonies were rod shaped, size ranging from 2-5 mm in diameter. Bacterial isolates were motile, texture was mucoid and transparent, and dew drop appearance was observed. Out of 10 isolates, 4 isolates C5, T7, L9 and M10 showed cyst formation.

The screening of 10 *Azotobacter* isolates for plant growth promoting activities were also carried out in which Indole Acetic Acid (IAA), ammonia production, phosphate solubilization and nitrate reduction test were performed (Table 2). All the 10 isolates were able to produced IAA, Ammonia and reduced nirate while the C5, H3 isolates were able to solubilize phosphate.

The production of IAA by isolates was also estimated (Figure 1) by comparing with a standard graph of IAA. In the present study it was observed that isolate N6 showed highest IAA production which was 52µg/mL followed by P1(51µg/mL), H3(39µg/mL), C5(38µg/mL), T7(35µg/mL), M10(34µg/mL), I8(33µg/mL), Al2(15µg/mL), L9(12µg/mL) and R4(11µg/mL).

In the present study the stress tolerance ability of the isolates were also studied to some factors viz.- temp, pH, salt tolerance. The effect of temperature on the growth of isolates was checked. It was found that all the isolates showed optimum growth at room temperature followed by 37°C and 40°C temperature while at 15°C and 60°C very less or no growth was recorded. The effect of pH on the growth of isolates was checked. It was observed that isolates Al2, R4, C5, T7, I8 showed optimum growth at pH 9, isolates P1, N6 showed optimum growth at pH 5, L9 isolate showed optimum growth at pH 6, isolate H3 showed optimum growth at pH 8 while the 7 was the optimum pH for growth of isolate M10. The salt (NaCl) tolerance ability of all the isolates were also checked from 2 to 10% concentrations. It was found that isolates P1 was able to grow even at 10% of NaCl concentration, while isolates Al2, H3, N6 and L9 can tolerate and was able to grow at 8% NaCl

concentration. Isolates R4, C5, T7, I8, M10 were unable to tolerate the salt concentration.

Effects of seed germination by 10 isolates on seeds of moth bean (*Vigna aconitifolia*) were observed. The speed of seed germination, development of roots and shoots of the seed by bioinoculant of *Azotobacter* isolates used as a biofertilizer were effective and showed maximum growth as compared to the control. Maximum growth was recorded for the isolate H3 (19.2 cm) followed by C5 (17.9 cm), I8 (17.4 cm), P1 (16.8 cm), N6 (16.7 cm), T7 (16.2 cm), L9 (15.2 cm), M10 (14.9 cm), R4 (14.3 cm) and Al2 (12.2 cm). The growth of control was measured 11.5 cm.

**Table No. 1 : Morphological, Cultural and Biochemical Characteristics of Isolates**

| Characteristics | Isolates | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | P1 | Al2 | H3 | R4 | C5 | N6 | T7 | I8 | L9 | M10 |
| Gram character | - | - | - | - | - | - | - | - | - | - |
| Motility | + | + | + | + | + | + | + | + | + | + |
| Cyst formation | - | - | - | - | + | - | + | - | + | + |
| Colony shape | Rod | Rod | Rod | Rod | Rod | Rod | Rod | Rod | Rod | Rod |
| Size (mm) | 2.8 | 3.8 | 3.0 | 4.2 | 4.2 | 5.6 | 2.8 | 5.0 | 3.0 | 2.5 |
| Colour | Off white | Off white | Creamy white | Creamy white | Off white | Off white | Off white | Creamy white | Creamy white | Off white |
| Elevation | Convex | Flat | Convex | Convex | Convex | Flat | Flat | Convex | Convex | Convex |
| Margin | Entire | Entire | Entire | Entire | Entire | Entire | Entire | Entire | Entire | Entire |
| Opacity | Opaque | Opaque | Opaque | Opaque | Opaque | Opaque | Opaque | Opaque | Opaque | Opaque |
| Texture | Mucoid | Mucoid | Mucoid | Mucoid | Mucoid | Mucoid | Mucoid | Mucoid | Mucoid | Mucoid |
| Indole test | + | + | + | + | + | + | + | + | + | + |
| Methyl red test | + | + | + | + | + | + | + | + | + | + |
| Vogus praskaur test | - | - | - | - | - | - | - | - | - | - |
| Citrate utilization test | + | + | + | + | + | + | + | + | + | + |
| Catalase | + | + | + | + | + | + | + | + | + | + |
| Oxidase | + | + | + | + | + | + | + | + | + | + |

| test | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Urease test | + | + | + | + | + | - | + | - | + | + |
| Amylase | - | - | - | - | - | - | - | - | - | - |
| Gelatinase | - | - | - | - | - | - | - | - | - | - |
| Glucose | - | + | + | + | + | + | - | - | - | - |
| Fructose | - | + | + | + | + | - | - | - | - | - |
| Lactose | + | + | - | - | + | + | - | - | - | - |
| Mannitol | - | + | + | + | + | - | - | - | - | - |
| Sucrose | - | + | + | + | + | - | - | - | - | - |
| Nitrate Reduction | + | + | + | + | + | + | + | + | + | + |

**Table No. 2 : Screening of *Azotobacter* isolates for plant growth promoting activities.**

| Test | Isolates | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $P_1$ | $Al_2$ | $H_3$ | $R_4$ | $C_5$ | $N_6$ | $T_7$ | $I_8$ | $L_9$ | $M_{10}$ |
| Indole Acetic Acid | + | + | + | + | + | + | + | + | + | + |
| Phosphate solubilization | - | - | + | - | + | - | - | - | - | - |
| Ammonia production | + | + | + | + | + | + | + | + | + | + |
| Nitrate reduction | + | + | + | + | + | + | + | + | + | + |

**Fig 1: Production of IAA by Isolates**

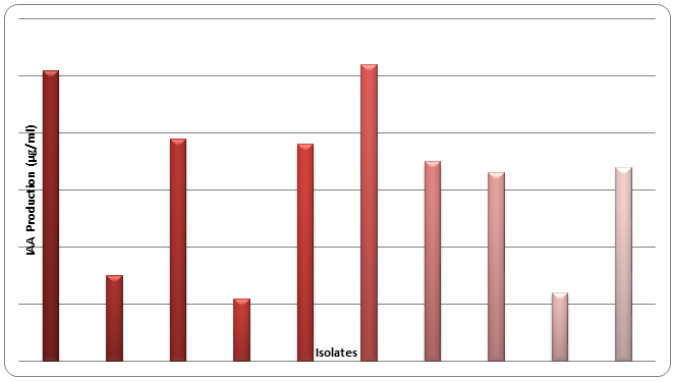



**Fig 3: Determination of pH tolerance by isolates**

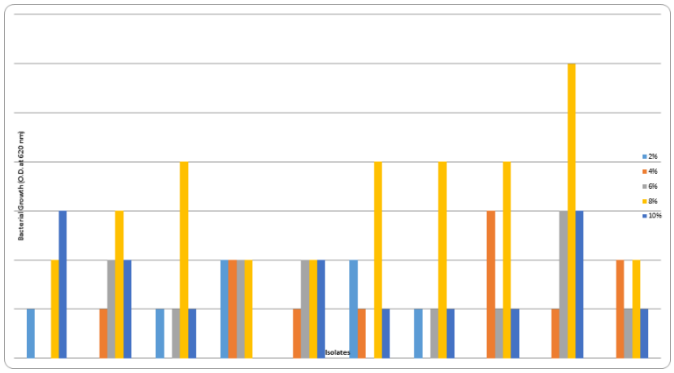



**Fig 2: Determination of temperature tolerance by isolates**

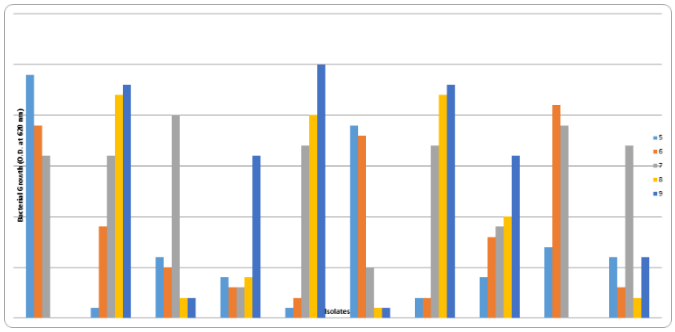


**Figure 4: Determination of salt tolerance by isolates**

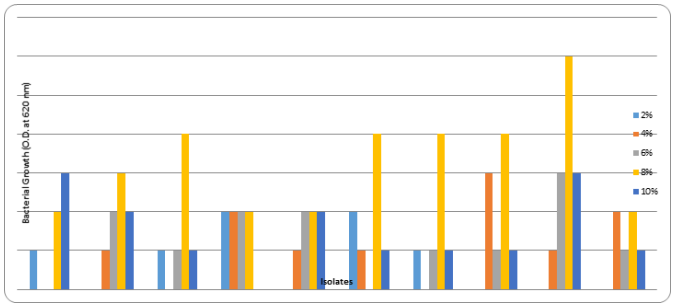
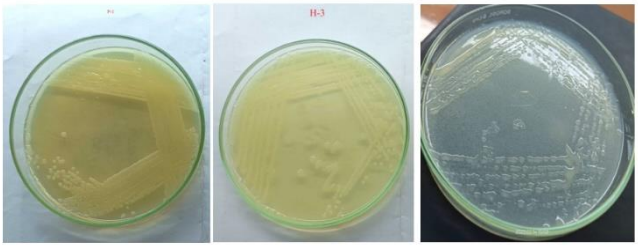


**Fig 5: Isolation of *Azotobacter spp* on Azotobacter Isolation Agar and Burks Medium**



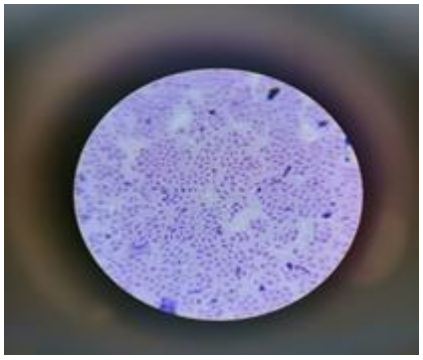


**Fig 6: Cyst formation**


**Fig 7: Phosphate Solubilization Test**

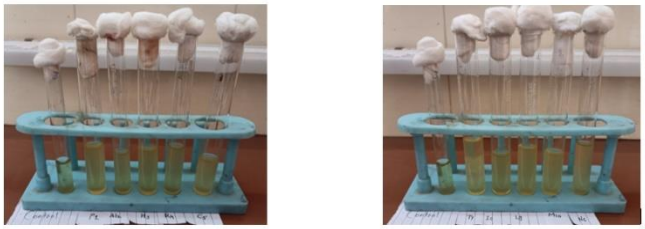


**Fig 8: Ammonia Production Test**

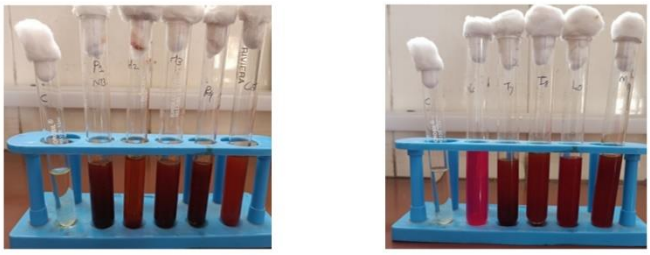
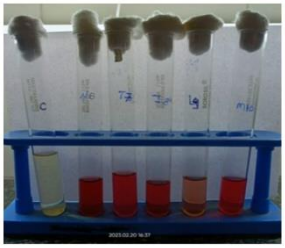


**Fig 9: Nitrate Reduction Test**



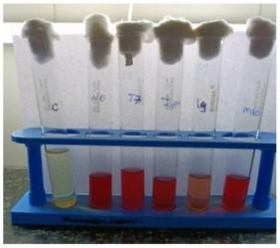


**Fig 10: IAA Production by isolates**


**Fig 11: Seed Germination**

## IV. DISCUSSION

In the present study, 10 *Azotobacter species* were isolated from soil samples randomly collected from Akola and Akot region for its possible use in crop improvement. Several other studies like Chen *et al.*, (2018); Shaikh and Shakir (2018); Andhare *et al.*, (2019) also reported the isolation of *Azotobcter species* from soil samples of different places and fields. Plant growth promoting activities such as Indole Acetic Acid production, phosphate solubilization, ammonia production, nitrate reduction of the ten isolates were studied. On pikovskaya media, a clear zone was formed around the colony by two isolates H3 and C5, this indicated that the isolate was able to solubilize phosphate. A study by Hafez *et al.,* (2016) demonstrated that *A. vinelandii* strain was able to solubilize up to 43% of the Abu Tartur phosphate rock in Egypt. All the isolates were able to reduced nitrate to nitrites. Akhter *et al.,* (2012); Samal *et al.,* (2020) also reported nitrate reduction by *Azotobacter* isolates. Ammonia production test was also performed, all 10 isolates were able to produced ammonia. Similarly Samal *et al.,* (2020) also performed the ammonia production test by

*Azotobacter species* and reported positive results for the isolates.

One of the important, plant growth promoting trait showed by *Azotobacter species* is auxin (IAA) production. This phytohormone helps the production of longer roots and increases number of root hairs and lateral roots which are involved in nutrient uptake (Datta and Basu, 2000). It plays a central role in cell division, elongation, fruit development and senescence. To determine the quantity of IAA produced a standard graph was prepared with different concentrations of IAA. The Indole Acetic Acid by all 10 isolates were produced (Figure 1). Several other studies have also reported the IAA production in *Azotobacter* isolates (Samal *et al.*, 2020; Purwaningsih *et al.*, 2022; Jain *et al.,* 2021).

The isolates obtained were further checked for their survival and stress tolerance capabilities for temperature (Figure 2), pH (Figure 3) and salt (NaCl) concentration (Figure 4) and compared with negative control. From the results it was found that all the isolates were survived in temperature ranges from room temperature to 40°C. Maximum growth was at room temperature followed by 37°C and even to 40°C while no growth was observed at 15°C and 60°C. This is in accordance with the studies of Samal *et al.*, (2020) who also reported maximum growth of *Azotobacter species* at 30°C and 37°C and no growth at 10°C and 50°C temperature. Similarly, Andhare *et al.*, (2019) also reported growth of *Azotobacter* at 28°C, 37°C and 40°C but no growth was found above 45°C temperature. Gomare *et al.,* (2013) also reported the cultivation of *Azotobacter* at 30°C.

Regarding the maximum growth of 10 isolates for different pH, for five isolates (Al2, R4, C5, T7, I8) optimum pH for growth was 9, two isolates (P1, N6) showed optimum growth at pH 5, one isolate (L9) showed optimum growth at pH 6, other one isolate (M10) showed optimum growth at pH 7 while one isolate (H3) showed optimum growth at pH 8. Samal *et al.*, (2020) also checked the growth of *Azotobacter* isolates for pH (5, 6, 7, 8, 9) they reported four isolates showed maximum growth at pH 6 followed by pH 9 while the other one isolate showed highest growth at pH 9 followed by pH 6.

The ability to tolerate the salt concentration by isolates was also studied in which R4, C5, T7, I8, M10 isolates were unable to tolerate the salt concentration from 2 to 10% while the isolates Al2, H3, N6, L9 can tolerate upto 8% NaCl concentration and P1 was able to grow even at 10% salt concentration. Akhter *et al.*, (2012) also demonstrated the growth of *Azotobacter* isolates in different salt concentration in which all the isolates showed growth at 0%, 2%, 4% NaCl concentration. 10 isolates showed no growth at 6% salt concentration and only 5 isolates showed negligible growth at 6% salt concentration. All isolates showed no growth at 10% salt concentration except 2 isolates survived in 10% salt concentration while 5 isolate were resistance to 6% salt concentration. Samal *et al.*, (2020) revealed that as there is increase concentration of the salt from 2 to 10% the growth decreased or may be no growth by *Azotobacter* isolates. Islam *et al.*, (2008), also studied the salt tolerance ability of isolates. It as found that all 6 isolates showed optimum growth salt concentration 0.0%, while poor growth was observed at salt concentration 0.2% followed by 0.4%. Only two isolates survived at salt concentration 0.6% and 0.8% while no growth was found at 1.0% salt concentration.

The genus *Azotobacter* has been used as a biofertilizer more than a century (Gerlach and Vogel, 1902). The effect of seed germination on moth beans using N - free Mannitol broth inoculated by ten isolates were also observed. Seeds treated with *Azotobacter* increased the seed germination rate and increased growth of root and shoot were reported. The isolate H3 showed highest growth (19.2 cm) compared to low growth was recorded in control. The effect of liquid formulation of *Azotobacter spp.* inoculation on germination and growth parameters of *Vigna radiata* (Mung beans) was also studied by Andhare *et al.,* (*20*19). Similar type of result was also reported by

Sharma *et al.,* (2007) in okra due to inoculation with PSB in *Cicer arientinum* L in seed. Pathak *et al.,* (2013) and Mahato *et al.,* (2009) reported significant role of *Azotobacter species* in increasing seed germination. Mukhter *et al*., (2018), studied the effect of biofertilizer on the growth of Maize Seedlings (Roots size).

## V. CONCLUSION

In the present study, 25 soil samples were collected from different places of Akola and Akot region. Out of 25 soil samples, 10 isolates were isolated of *Azotobacter species.* All the isolates were able to produced IAA, Ammonia and two isolates solubilized the phosphate. All the 10 bacterial isolates was capable to reduced the nirate. The effect of growth on *Azotobacter* isolates of various stress parameters viz. temperature, pH, salt concentration were also studied while all isolates showed maximum growth at room temperature followed by 37°C, 40°C while some isolates showed maximum growth tolerating varied pH and salt. Effective result of seed germination by 10 isolates on seed were observed. Biofertilizers of such *Azotobacter spp. may be* helpful for farmers to increase the crop productivity as biofertilizers are eco-friendly and can replace the use of chemical fertilizers.

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