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ISOLATION AND CHARACTERIZATION OF SOIL MENDING *Azotobacter spp.* FROM RHIZOSPHERE OF CHILLI (*Capsicum annum* L.)

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KEYWORDS

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Brown pigment

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ABSTRACT

Seventy five *Azotobacter sp* were isolated from rhizospheric soils of chilli grown in Raichur, Koppal, Bellary, Yadagiri and Kalaburagi region (parts of Hyderabad Karnataka) and characterized by using both morphological and biochemical tests. Compound Microscopic observation showed that all the isolates were rod shaped and Gram-negative. These isolates were capable of producing mildly heat and stress resistant cyst under nutrient deprivation and physical stress conditions. Interestingly, light brown to dark brown pigmentation varying with isolate are observed after 7 to 14 days after incubation at 37 ^{0}C. These isolates were capable of utilizing Protein (gelatin), starch, tryptophan whereas, production of ammonia is observed at the end of protein degradation. Most of the isolates were capable of reducing nitrate, a positive sign for the fixation of free nitrogen.

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1 Introduction

Azotobacter is a Gram-negative bacterium belongs to family *Azotobacteracea*. Most of the members of this family belongs to gamma subclass of *Proteobacteria* and have capability of free nitrogen fixation by utilizing nitrogenase enzyme (Komagata et al., 2014). Member of the genus *Azotobacter* are pleomorphic (bacillary to spherical shape) and their size ranges from 1.0 to 3.8 µm. Many of the *Azotobacter sp* produces exaggerated amounts of pigments varying from black to brown (*A. chroococcum*), pinkish (*A. macrocytogenes)*, greenish fluorescent (*A. vinelandii*, *A. agilis*, and *A. paspali*), grayish blue (*A. insignis*) and yellow (*A. beijerinckii*) (Jiménez et al., 2011). This pigmentation protects these bacteria from UV, desiccation, Superoxide radicals, and drought. In addition, under adverse conditions member of genus *Azotobacter* produces cysts, which are resistant to mild heat and desiccation (Orville et al., 1961) and provides a comparative advantage to compete in rhizosphere with pathogens. During the process of encystment respiration rate is comparatively decreased (Socolofsky & Orville, 1962) however, they can metabolize the carbon (Orville et al., 1961).

Azotobacter sp. commonly dispersed in neutral to alkaline soils (Aquilanti et al., 2004); widely grow at optimum temperature range of 28- 32 ºC. Roots are usually teeming with menagerie of microbes; root colonization is one of the most important steps in the interaction of bacteria and host plants (Narula et al., 2007). These bacteria have proved to be beneficial for the plants by directly nitrogen fixation, phosphate solubilization, antibiosis, inducing systemic resistance, phytohormone production, lowering ethylene concentration. In PGPR bacteria, *Azotobacter* have specific place and frequently used to enhance the growth and yield of plants.

Chilli (*Capsicum annum* L.) is an important vegetable crop, and its socio-cultural role is remarkable worldwide and it is an indispensable condiment of Indian cuisine because of its pungency, color, flavor and aroma. Continuous use of chemical fertilizers disrupts soil ecology and environment; degrades soil fertility, which leads to harmful effects on human and animal health (Ayala & Rao, 2002); and contaminates groundwater resources (Joshi et al., 2006). Further, the use of chemical fertilizers and pesticides to control plant diseases and pathogens has decreased fertility status of the soil and created unhealthy environment. In response to this, researchers from worldwide have focused their effort to develop alternative measures to synthetic chemicals for promoting plant growth and yield. This study aims to isolate and to characterize the *Azotobacter* isolates from rhizospheric soils of chilli grown in HK region (Hyderabad Karnataka regions of India). Characterization of isolated isolates carried out by morphological and biochemical tests, which gives a brief idea regarding prevalence of *Azotobacter sp* in dry land regions of Karnataka. It is imperative to understand the situation before proceeding to solve the problem hence we tried to explore the presence of beneficial organisms near rhizosphere as much as possible.

2 Material and methods

2.1 Collection of soil samples

A total of one fifty (150) chilli rhizospheric soil samples were collected from the different districts of HK regions (Hyderabad Karnataka) including Raichur (16.18'23.3'N 77°9'20'E), Koppal (15.35° N 76.15°E), Bellary (15°06'N76°55'E), Yadagiri (16.77°N 77.13° E) and Kalaburagi (17.12° N 76.31° E). For soil sample collection, top 15 cm of soil carefully collected by using sterile soil scoop and packed immediately by using sterile polythene cover. Samples placed in the deep freezer (below -15 ^{0}C) and processed next day for isolation and characterization of *Azotobacter species*.

2.2 Isolation of *Azotobacter sp*

Azotobacter sp were isolated from rhizospheric soils of chilli by serial dilution plate technique. Soil samples serially diluted up to 10^{-4} as per the standard protocol, from 10^{-4} dilution an aliquot of 0.1 ml of uniformly mixed soil suspension aseptically spread on Waksman No.77 N-free agar medium (Mannitol: 10.0 g , CaCO3: 5.0 g, K_2HPO_4: 0.5 g, $MgSO_4$: 0.2 g, NaCl: 0.01 g, Agar: 20 g, Distilled water: 1000 ml and pH: 7.0) (Allen, 1953). Thereafter the inoculated plates incubated at 30 ^{0}C for 4-6 days in standard BOD incubator.

2.3 Morphological characterization of *Azotobacter* isolates

All the isolates of *Azotobacter* were examined for their cell morphology, colony morphology and gram reaction as per the standard procedures given by Cappuccino & Sherman (1992).

2.4 Cyst formation

The *Azotobacter* isolates cultured for 7 days on Waksman No. 77 (Nitrogen free) agar medium. Individual culture was picked aseptically on to the clean grease and oil free slide and stained with Violamine stain for 45 seconds followed by washing and counter staining with crystal violet to observe the presence of cysts (Winogradsky, 1949).

2.5 Biochemical characterization of *Azotobacter* isolates

Biochemical characterizations of *Azotobacter sp* were carried out by employing the standard procedures given by Cappuccino & Sherman (1992). Different biochemical tests performed for *Azotobacter sp* are briefly outlined below.

2.5.1 Catalase test

Azotobacter isolates aseptically inoculated on labeled nutrient agar slants by streak and incubated at 30 ^{0}C for 24 h. After incubation, the slants were flooded with 3-4 drops of 3 per cent (%) hydrogen peroxide and examined for the production of bubbles or foam (Cappuccino & Sherman, 1992).

2.5.2 Starch hydrolysis

Organisms capable of hydrolyzing starch to maltose possess the enzyme amylase. By this test, the presence or absence of this enzyme in the organisms was ascertained. *Azotobacter* isolates streaked on the starch agar plates and incubated at 37^{0}C for 24 h, then iodine solution was flooded on plates for 5 to 10 min. Plates showing halo clear zone near colonies are considered as positive whereas plates without halo zone are considered as negative for the test (Cappuccino & Sherman, 1992).

2.5.3 Indole production test

Ability to hydrolyze tryptophan with the production of indole is not a characteristic of all microorganisms and therefore serves as biochemical marker. For this test SIM agar supplemented with tryptophan is inoculated with individual bacterial test culture in respectively labeled tubes thereafter tubes incubated at 37 ^{0}C for 48 h. Presence of indole is detected by adding 1 ml of Kovac's reagent to each tube, formation of cherry red color ring on the top of the tube is positive test.

2.5.4 Gelatin liquefaction

Pre-sterilized nutrient gelatin deep tubes inoculated with *Azotobacter* isolates and incubated at 28^0 C for 24 h. After incubation, the tubes were kept in a refrigerator at 4^{0}C for 30 min. The tubes with *Azotobacter* isolates remained liquefied are taken as positive for the test (Cappuccino & Sherman, 1992).

2.5.5 Nitrate reduction

Azotobacter isolates inoculated in to the pre-sterilized trypticase nitrate semisolid medium (supplemented with 1 % KNO_3) and kept it for incubation at 37 ^{0}C for 48 h. Addition of three drops of α-napthylamine and one drop of sulphanilic acid results in development of red color indicates positive for the test whereas no color change for negative for the test (Cappuccino & Sherman, 1992).

2.5.6 Ammonification

Pure culture of *Azotobacter* isolates were inoculated into tubes containing 10 ml peptone water and incubated at 30^0 C for 48 h. After incubation, Nessler′s reagent (0.5 ml) was added to each tube (Cappuccino & Sherman, 1992) and the tubes were observed for the development of yellow color.

3 Results and Discussion

3.1 Isolation of *Azotobacter* isolates

From the collected 150 rhizospheric soil samples, total of 75 overtly grown *Azotobacter* isolates were isolated from rhizospheric soil of chilli after 4-6 days of incubation. All the 75 *Azotobacter* isolates showed small/medium, milky white, round, raised colonies on Waksman No. 77 plates (Figure: 1) during early growth. Colonies have slow growth up to 2 days later on growth was very fast (Wu et al., 2006).

3.2 Morphological characterization of *Azotobacter* isolates

All the collected 75 *Azotobacter* isolates were taken for the morphological characterization. Result of morphological identification revealed Gram negative, rod shaped, forming white

Figure 1 Cultures of *Azotobacter* isolate on Waksman No. 77 plates

raised, wrinkled edge colony on Waksman No. 77 medium. Pigment production differs with individual isolate as dark brown and light brown (Table 1; Figure 1) (Jacobson, 2000).These observations are in agreement with the characteristics of *Azotobacter* described in Bergey's manual of determinative bacteriology (Brenner et al., 2005). The formation of brown insoluble

Table 1 Morphological and biochemical characteristics of *Azotobacter* isolates isolated from Chilli Rhizosphere soil of Hyderabad Karnataka region

Sl. No.	Isolate code	Cell shape	Motility	Colony color	Gram reaction	CT	SH	GL	NR	A	IT
1	AZT-J1	Rod	Motile	Light brown	Negative	+	+	+	+	+	+
2	AZT-J2	Rod	Motile	Light brown	Negative	+	+	+	-	+	+
3	AZT-J3	Rod	Motile	Light brown	Negative	+	+	+	-	+	+
4	AZT-J4	Rod	Motile	Brown	Negative	+	+	+	+	+	+
5	AZT-J5	Rod	Motile	Dark brown	Negative	+	+	+	-	+	+
6	AZT-J6	Rod	Motile	Dark brown	Negative	+	+	+	+	+	+
7	AZT-J7	Rod	Motile	Dark brown	Negative	+	+	+	-	+	+
8	AZT-J8	Rod	Motile	Dark brown	Negative	+	+	+	-	+	+
9	AZT-J9	Rod	Motile	Light brown	Negative	+	+	+	-	+	+
10	AZT-J10	Rod	Motile	Light brown	Negative	+	+	+	+	+	+
11	AZT-J11	Rod	Motile	Light brown	Negative	+	+	+	-	+	+
12	AZT-J12	Rod	Motile	Dark brown	Negative	+	+	+	-	+	+
13	AZT-J13	Rod	Motile	Dark brown	Negative	+	+	+	-	+	+
14	AZT-J14	Rod	Motile	Dark brown	Negative	+	+	+	+	+	+
15	AZT-J15	Rod	Motile	Light brown	Negative	+	+	+	-	+	+
16	AZT-Y1	Rod	Motile	Light brown	Negative	+	+	+	-	+	+
17	AZT-Y2	Rod	Motile	Dark Brown	Negative	+	+	+	-	+	+
18	AZT-Y3	Rod	Motile	Dark Brown	Negative	+	+	+	-	+	+
19	AZT-Y4	Rod	Motile	Light brown	Negative	+	+	+	+	+	+
20	AZT-Y5	Rod	Motile	Light brown	Negative	+	+	+	-	+	+
21	AZT-Y6	Rod	Motile	Dark Brown	Negative	+	+	+	+	+	+
22	AZT-Y7	Rod	Motile	Brown	Negative	+	+	+	+	+	+
23	AZT-Y8	Rod	Motile	Brown	Negative	+	+	+	+	+	+
24	AZT-Y9	Rod	Motile	Light brown	Negative	+	+	+	+	+	+
25	AZT-Y10	Rod	Motile	Light brown	Negative	+	+	+	-	+	+
26	AZT-Y11	Rod	Motile	Brown	Negative	+	+	+	-	+	+
27	AZT-Y12	Rod	Motile	Light brown	Negative	+	+	+	-	+	+
28	AZT-Y13	Rod	Motile	Light brown	Negative	+	+	+	+	+	+
29	AZT-Y14	Rod	Motile	Dark Brown	Negative	+	+	+	+	+	+
30	AZT-Y15	Rod	Motile	Dark Brown	Negative	+	+	+	-	+	+
31	AZT-R1	Rod	Motile	Brown	Negative	+	+	+	-	+	+
32	AZT-R2	Rod	Motile	Brown	Negative	+	+	+	+	+	+
33	AZT-R3	Rod	Motile	Light brown	Negative	+	+	+	-	+	+
34	AZT-R4	Rod	Motile	Light brown	Negative	+	+	+	-	+	+
35	AZT-R5	Rod	Motile	Brown	Negative	+	+	+	-	+	+
36	AZT-R6	Rod	Motile	Light brown	Negative	+	+	+	+	+	+

Sl. No.	Isolate code	Cell shape	Motility	Colony color	Gram reaction	CT	SH	GL	NR	A	IT
37	AZT-R7	Rod	Motile	Light brown	Negative	+	+	+	-	+	+
38	AZT-R8	Rod	Motile	Dark brown	Negative	+	+	+	-	+	+
39	AZT-R9	Rod	Motile	Dark brown	Negative	+	+	+	+	+	+
40	AZT-R10	Rod	Motile	Dark brown	Negative	+	+	+	-	+	+
41	AZT-R11	Rod	Motile	Light brown	Negative	+	+	+	-	+	+
42	AZT-R12	Rod	Motile	Light brown	Negative	+	+	+	-	+	+
43	AZT-R13	Rod	Motile	Dark brown	Negative	+	+	+	+	+	+
44	AZT-R14	Rod	Motile	Light brown	Negative	+	+	+	-	+	+
45	AZT-R15	Rod	Motile	Light brown	Negative	+	+	+	-	+	+
46	AZT-G1	Rod	Motile	Dark Brown	Negative	+	+	+	+	+	+
47	AZT-G2	Rod	Motile	Brown	Negative	+	+	+	+	+	+
48	AZT-G3	Rod	Motile	Light brown	Negative	+	+	+	+	+	+
49	AZT-G4	Rod	Motile	Light brown	Negative	+	+	+	+	+	+
50	AZT-G5	Rod	Motile	Dark brown	Negative	+	+	+	-	+	+
51	AZT-G6	Rod	Motile	Dark brown	Negative	+	+	+	-	+	+
52	AZT-G7	Rod	Motile	Dark brown	Negative	+	+	+	-	+	+
53	AZT-G8	Rod	Motile	Dark brown	Negative	+	+	+	-	+	+
54	AZT-G9	Rod	Motile	Dark brown	Negative	+	+	+	-	+	+
55	AZT-G10	Rod	Motile	Dark brown	Negative	+	+	+	+	+	+
56	AZT-G11	Rod	Motile	Light brown	Negative	+	+	+	+	+	+
57	AZT-G12	Rod	Motile	Light brown	Negative	+	+	+	-	+	+
58	AZT-G13	Rod	Motile	Dark brown	Negative	+	+	+	-	+	+
59	AZT-G14	Rod	Motile	Light brown	Negative	+	+	+	+	+	+
60	AZT-G15	Rod	Motile	Dark Brown	Negative	+	+	+	-	+	+
61	AZT-B1	Rod	Motile	Brown	Negative	+	+	+	-	+	+
62	AZT-B2	Rod	Motile	Light brown	Negative	+	+	+	+	+	+
63	AZT-B3	Rod	Motile	Light brown	Negative	+	+	+	-	+	+
64	AZT-B4	Rod	Motile	Light brown	Negative	+	+	+	-	+	+
65	AZT-B5	Rod	Motile	Dark Brown	Negative	+	+	+	-	+	+
66	AZT-B6	Rod	Motile	Brown	Negative	+	+	+	-	+	+
67	AZT-B7	Rod	Motile	Light brown	Negative	+	+	+	+	+	+
68	AZT-B8	Rod	Motile	Light brown	Negative	+	+	+	+	+	+
69	AZT-B9	Rod	Motile	Light brown	Negative	+	+	+	-	+	+
70	AZT-B10	Rod	Motile	Dark Brown	Negative	+	+	+	+	+	+
71	AZT-B11	Rod	Motile	Brown	Negative	+	+	+	+	+	+
72	AZT-B12	Rod	Motile	Light brown	Negative	+	+	+	-	+	+
73	AZT-B13	Rod	Motile	Light brown	Negative	+	+	+	+	+	+
74	AZT-B14	Rod	Motile	Light brown	Negative	+	+	+	-	+	+
75	AZT-B15	Rod	Motile	Dark Brown	Negative	+	+	+	+	+	+

CT-Catalase test, SH-Starch hydrolysis, GL-Gelatin liquefaction, NR-Nitrate reduction, A-Ammonification, IT-Indole test

Figure 2 Presence of bubbling/ foaming after addition of hydrogen peroxide in the nutrient agar slants inoculated with *Azotobacter* isolates indicating positive for the catalase test

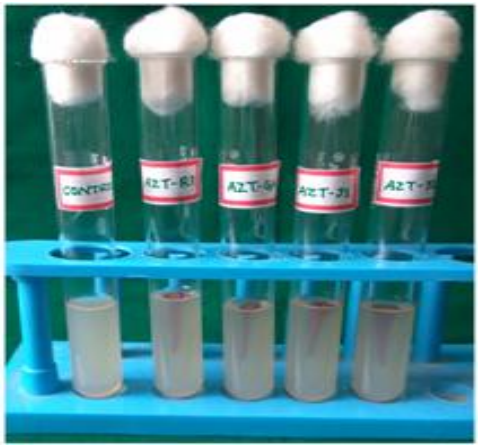

Figure 3 Motility test positive for *Azotobacter* isolates

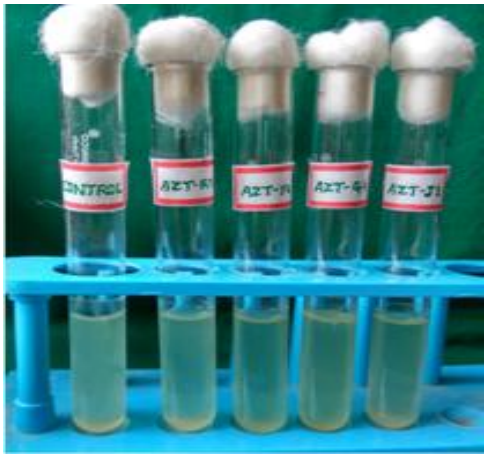

Figure 4 No formation of red color in nitrate broth inoculated with efficient *Azotobacter* isolates up on addition of nitrate test reagent indicating negative for the test

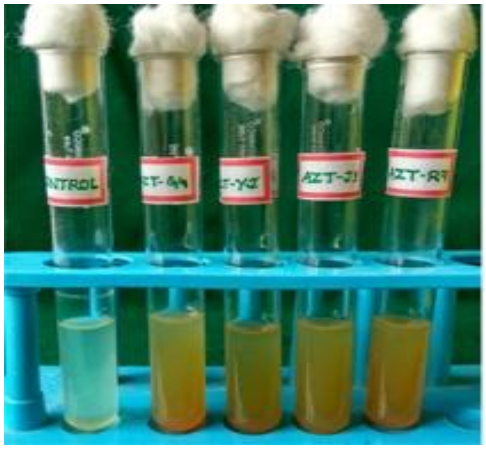

Figure 5 Yellow coloration in peptone broth upon addition of Nessler's reagent inoculated with efficient *Azotobacter* isolates indicating positive for ammonification process

pigments is characteristic of species *A. chroococcum* (Gibbs & Shapton, 1968). All the isolates formed cysts under nutrient and physical stress conditions. Earlier reports shows that *Azotobacter* can survive in the form of cysts up to 24 years in dry soil conditions and the formation of cyst is the unique identification characters of genus *Azotobacter* (Hitchins & Sadoff, 1973; Page & Shivprasad, 1991; Moreno et al.,1999).

3.3 Biochemical characterization of efficient *Azotobacter* isolates

All the *Azotobacter* isolates showed positive results for catalase test, starch hydrolysis gelatin liquefaction, ammonification, and indole test. Few isolates were negative for nitrate reduction. In this manner result of present study are in agreement with the findings of previous researchers (Juarez et al., 2005; Murumkar et al, 2012; Jenifer et al., 2013; Kumar et al., 2014) (Table 1 and Figure 2,3,4 and 5).

Conclusion

Our study aimed in the isolation and characterization of isolates from northern districts of Karnataka. Usually these districts fall under dry lands or rain fed areas, which is a major deterrent for the growth of crops, which require plethora of water and fertilizer. Promotion of water conservation and limited fertilizer application is impeccably practical. Most of the *Azotobacter* isolates can fix nitrogen and conserves the moisture by producing excess exopolysaccharides. In conjunction with this, these *Azotobacter sps.*, also promote plant growth by several other mechanisms like phosphate solubilization, antagonism, production of phytohormone, and degradation of toxic compounds. In the present investigation, some isolates were able to reduce the nitrate under anaerobic conditions, which may help the plant by fixing

more nitrogen, or reduce the available nitrogen for the plant. Ammonification by all the isolates was favorably enhancing the plant growth. All isolates are producing cysts after the exponential phase of growth or under adverse conditions due to the lack of nutrients. Many of the isolates shown the production of copious amounts of brown to black color pigmentation, there is an inclined pigmentation was also observed as the maximum temperature increases with location. Pigmentation serves as protective agent against extreme heat and cold, UV, act as a cellular scavenger against free radicals, Reactive Oxygen Species (ROS), drugs, oxidents, xenobiotics and moreover melanized cells are resistant to certain antifungal too.

Azotobacter species maintains the natural habitat of the soil and increases crop yield by 20-30 %. It replaces chemical nitrogen and phosphorus by 25 % in addition to stimulating the plant growth. Finally, it can provide protection against drought and some soil borne diseases.

Conflict of interest

No potential conflict of interest was reported by the authors.

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