RESEARCH ARTICLE

ISOLATION AND CHARACTERIZATION OF NATIVE *AZOTOBACTER* ISOLATES FROM RHIZOSPHERIC SOIL SAMPLES

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Abstract: A total of thirty *Azotobacter* isolates were obtained and characterized on the basis of their colony morphology, microscopy and biochemical test. Isolates were repeatedly subcultured on *Azotobacter* agar (Mannitol) medium to obtain pure cultures of *Azotobacter*. All the isolates showed creamy translucent, mucoid, and circular shape colony morphology. Colonies having *Azotobacter* like morphology were microscopically analyzed and those depicting oval-rod shaped Gram negative bacteria were selected. All *Azotobacter* isolates were further characterized by different biochemical test. Isolates A-2, A-8, A-16, A-23, A-24 and A-28 showed positive results in all the biochemical tests (Triple sugar iron agar test, Citrate utilization test, Methyl red test, Voges-Proskauer test, Catalase test, Oxidase test,Nitrate reduction test, Urease test, Starch hydrolysis test and Motility test). Further, antibiotic sensitivity profiling of these isolates was done all the isolates were found resistant to Amoxyclav and Erythromycin and all were inhibited by the Ciprofloxacin by forming a clear zone of 15mm. All isolates were also tested for Phosphorus solublization activity on PVK medium and none of the isolates were able to solubilize phosphorus. These *Azotobacter* isolates were tested for physiological efficiency on different pH (6, 7, and 8). All isolates grew well on alkaline medium of pH value 8. Twenty five isolates grew well at pH 6 and 7. It was observed that A-13, A-17, A-19, and A-20 showed no growth at pH 7. Results showed that A-15, A-19 and A-20 were unable to grow at pH 6.

Keywords: Isolation, *Azotobacter*, Phosphorus solublization, Characterization

INTRODUCTION

Conventional agriculture plays a significant role in meeting the food demands of a growing human population, which has also led to an increasing dependence on chemical fertilizers and pesticides (Santos *et. al.*, 2012). Chemical fertilizers are industrially manipulated, substances composed of known quantities of nitrogen, phosphorus and potassium, and their exploitation causes air and ground water pollution by eutrophication of water bodies (Youssef *et. al.*, 2014). In general, 60% to 90% of the total applied fertilizer is lost and the remaining 10% to 40% is taken up by plants. In this regard, microbial inoculants have paramount significance in integrated nutrient management systems to sustain agricultural productivity and healthy environment (Adesemoye and Kloepper, 2009). Recent efforts have been channelized towards the production of 'nutrient rich high quality food' to ensure bio-safety. The innovative view of farm production attracts the growing demand of biological based organic fertilizers as promising alternative to agro-chemicals (Raja *et. al.*, 2013). In agriculture, encouraging alternate means of soil fertilization relies on organic inputs to improve nutrient supply and conserve the field management (Araujo *et. al.*, 2008). Such organic inputs will be of crucial importance to popularize organic farming including enhanced biodiversity of soil rhizosphere (Megali *et. al.*, 2013).

Among the biofertilizers nitrogen fixing microorganisms plays a crucial role to reduce the dependency on inorganic nitrogen sources. As Azotobacter is an important member of nitrogen fixing family which belongs to family *Azotobacteriaceae*, aerobic, free living, and heterotrophic in nature. *Azotobacter*s are present in neutral or alkaline soils and *A. chroococcum*is the most commonly occurring species in arable soils. The occurrence of above organism has been reported from the rhizosphere of a number of crop plants such as rice, maize, sugarcane, bajra, vegetables and plantation crops (Mishra *et. al.*, 2013). Keeping the above considerations in view it is of great importance to obtain its native isolates from various locations to characterize and identify the efficient isolates.

MATERIALS AND METHODS

Collection of Soil Samples

30 Soil samples were collected from different locations of Sardar Vallabhbhai Patel University of Agriculture & Technology, Modipuram, Meerut and Hapur (Uttar Pradesh) from cultivated land. Soil Samples were collected from a depth of 0-10 cm in polyethylene bags, stored at field moisture level and room temperature. The reference *Azotobacter* strain was procured from Division of Microbiology, IARI Pusa, New Delhi.

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Isolation of *Azotobacter*

The pure *Azotobacter* isolates were obtained on *Azotobacter* agar medium (Mannitol). Single colonies were picked up and were re-streaked on *Azotobacter* agar medium (Mannitol) for obtaining pure cultures. Slants of same medium were prepared for the long time preservation of the isolates to be further utilized.

Colony and Cell Morphology

The morphological characteristics were studied for all isolates including the IARI reference strain. The isolates were evaluated for different colony morphology viz. creamy, white, transparent, translucent, non-gummy, gummy or mucoid, circular shape etc. All the colonies of different isolates were subjected to stain by the method of Gram's staining, and observations under microscope at 100X**.**

Biochemical Characterization

The samples were examined for 10 different biochemical characteristics namely, Triple sugar iron agar test, Citrate utilization test, Methyl red test, Voges-Proskauer test, Catalase test, Oxidase test,Nitrate reduction test, Urease test, Starch hydrolysis test and Motility test with typical procedure.Triple sugar iron agar test is used to determine whether gram negative bacilli utilize glucose and lactose or sucrose fermentatively and produce H_2S. Phenol red and ferrous sulphate serves as indicator for acidification of medium and H_2S production respectively.

Citrate Utilization test is used to detect the ability of an organism to utilize sodium citrate as a sole source of carbon and ammonium salt as a sole source of nitrogen.Methyl Red (MR) test determines whether the microbe performs mixed acids fermentation when supplied glucose. Voges-Proskauer involves observation of red color reaction produced by appropriate culture media after treatment with potassium hydroxide. The active product in the medium formed by bacterial metabolism is acetyl methyl carbinol, a product of the butylenes glycol pathway.

Catalase test was used to determine the presence of catalase, an enzyme that catalyses the release of O_2 and H_2O from hydrogen peroxide (H_2O_2). The oxidase test was used to identify bacteria that produce cytochrome c oxidase, an enzyme of the bacterial electron transport chain. When present, the cytochrome c oxidase oxidizes the reagent (tetramethyl-p phenylenediamine) to (indophenols) purple color end product. Nitrate reduction test is used for the differentiation of members of Enterobacteriaceae on the basis of their ability to produce nitrate reductase enzyme that hydrolyze nitrate (NO_3^-) to nitrite (NO_2^-) which may then again be degraded to various nitrogen products like nitrogen oxide, nitrous oxide and ammonia (NH_3) depending on the enzyme system of the organism and the atmosphere in which it is growing.

Urease test involves hydrolysis of urea with the release of ammonia and carbon dioxide. The ammonia combines with carbon dioxide and water to form ammonium carbonate which turns the medium alkaline, turning the indicator phenol red from its original orange yellow color to bright pink. Starch hydrolysis test involves the bacterial ability to hydrolyse starch, by the amylase enzyme. While the starch forms dark blue color with iodine, its hydrolyzed products do not acquire such dark blue color with iodine.

Motility test was used to detect the ability of an organism to move by itself by means of propeller-like flagella unique to bacteria or by special fibrils that produce a gliding form of motility. Sulphide Indole Motility (SIM) medium is a combination differential medium that tests three different parameters, Sulfur Reduction, Indole Production and Motility. This media has a very soft consistency that allows motile bacteria to migrate readily through them causing cloudiness in the stabbed area.

Antibiotic sensitivity profiling test

In Antibiotic sensitivity test, the disc diffusion method was was used to identify the sensitivity for Ciprofloxacin (Cf), Cloxacillin (Cx), Co-Trimoxazole (Co), Tetracycline (T), Amoxyclav (Ac), Cephalexin (Cp), Clindamycin (Cd), Tetracycline (T) and Erythromycin (E) antibiotic.

pH Tolerance Test

For pH stress tolerance capacity, the *Azotobacter* isolates were tested on *Azotobacter* agar media plate with pH (6.0, 7.0, and 8.0) by spot inoculation from log phase culture. Plates were incubated at 28˚C for 48-72 hours.

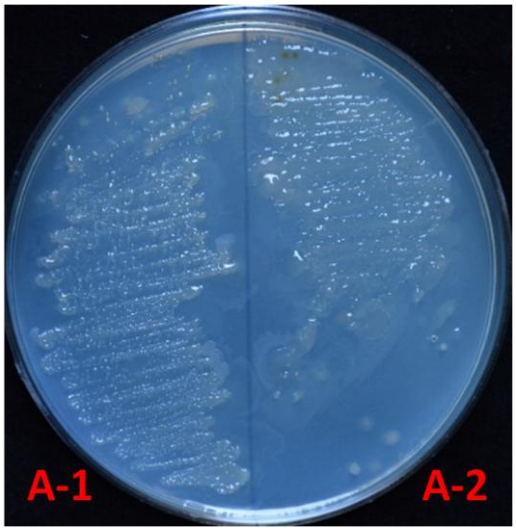

Figure 1. Colony morphology of pure culture of *Azotobacter* isolate (A-1 and A-2) on *Azotobacter* agar medium (Mannitol)

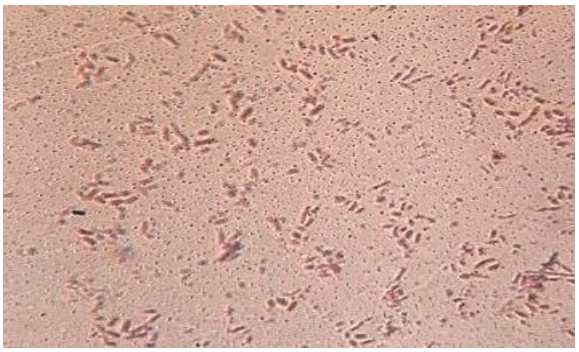

Figure 2. Microscopic analysis of *Azotobacter* isolate by Gram's staining.

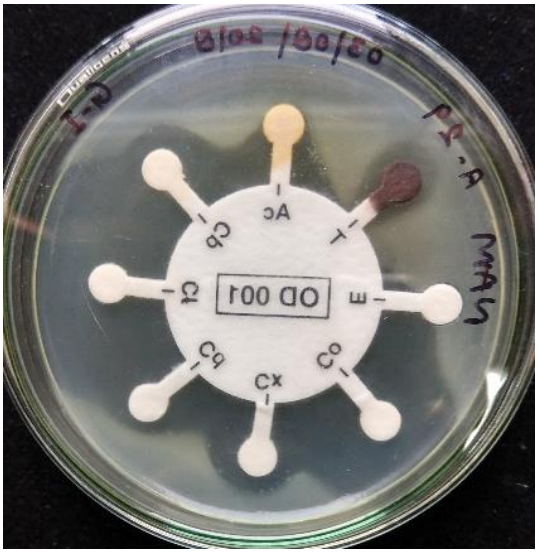

Figure 3. Octadisc method for antibiotic sensitivity profiling. Results show clear zone for specific antibiotics sensitivity.

Table 1. Studies on various biochemical tests for native *Azotobacter* isolates.

SAMPLE CODE	BIOCHEMICAL TESTS									
	TSI	**CU**	**MR**	**VP**	**CT**	**OT**	**NR**	**UT**	**SH**	**MT**
A-1	+	+	+	+	_	+	_	+	+	+
A-2	+	+	+	+	+	+	+	+	+	+

A-3	_	+	_	_	_	+	_	_	_	+
A-4	+	+	+	+	_	+	+	_	+	+
A-5	+	+	+	+	+	+	_	_	_	+
A-6	_	+	_	_	_	+	+	_	+	+
A-7	+	+	+	+	_	+	_	_	_	+
A-8	+	+	+	+	+	+	+	+	+	+
A-9	+	+	_	_	_	+	_	_	_	+
A-10	_	+	_	+	_	+	_	_	+	+
A-11	+	+	+	+	_	+	_	+	+	+
A-12	+	+	+	+	+	+	_	_	+	+
A-13	+	+	_	_	_	+	+	_	_	+
A-14	_	+	_	_	_	+	+	_	_	+
A-15	+	+	+	+	_	+	+	_	_	+
A-16	+	+	+	+	+	+	+	+	+	+
A-17	+	+	+	_	+	+	_	_	_	+
A-18	_	+	_	_	+	_	+	_	_	+
A-19	+	+	+	+	+	+	_	_	_	+
A-20	+	+	_	_	+	+	+	+	_	+
A-21	+	+	+	_	+	_	_	_	_	+
A-22	+	+	+	_	+	_	_	_	_	+
A-23	+	+	+	+	+	+	+	+	+	+
A-24	+	+	+	+	+	+	+	+	+	+
A-25	+	+	+	+	+	_	_	+	_	+
A-26	_	+	_	_	_	+	_	+	+	+
A-27	+	+	+	+	_	_	_	+	+	+
A-28	+	+	+	+	+	+	+	+	+	+
A-29	+	+	_	_	+	_	+	+	_	+
A-30	+	+	+	+	+	_	_	+	_	+
A-IARI	+	+	_	_	+	+	_	_	_	+

TSI= Triple sugar iron agar test, **CU=** Citrate utilization test, **MR=** Methyl red test, **VP=**Voges-Proskauer, **CT=** Citrate test, **OT=** Oxidase test, **NR=** Nitrate reduction test, **UT=** Urease test, **SH=** Starch hydrolysis test, **MT=** Motility test.

Table 2. Growth of different *Azotobacter isolates* at different pH medium.

S.No	Sample codes	Growth Medium		
		pH 6	pH 7	pH 8
1.	A-1	+	++	++
2.	A-2	+++	++	+++
3.	A-3	+	++	++
4.	A-4	++	++	++
5.	A-5	+++	+++	+++

6.	A-6	++	++	++
7.	A-7	+++	+++	+
8.	A-8	+++	++	++
9.	A-9	++	+	+
10.	A-10	+	++	+++
11.	A-11	++	++	+++
12.	A-12	+++	++	++
13.	A-13	++	_	++
14.	A-14	+	++	+
15.	A-15	_	+	++
16.	A-16	++	++	++
17.	A-17	++	_	+++
18.	A-18	+	+	++
19.	A-19	_	_	+
20.	A-20	_	_	+
21.	A-21	++	++	+
22.	A-22	+	++	+++
23.	A-23	++	++	+
24.	A-24	++	+	++
25.	A-25	+++	++	++
26.	A-26	++	++	+
27.	A-27	+++	+++	+++
28.	A-28	+	++	++
29.	A-29	+++	+++	+++
30.	A-30	+	+	+
31.	IARI Strain	+++	+++	+++

+++ =Best Growth, ++ = Good Growth, + = Less Growth, - = No

RESULTS AND DISCUSSION

A total of Thirty soil samples were cultured on *Azotobacter* agar medium (Mannitol). The pure *Azotobacter* isolates were identified based on their colony morphology (Tchan *et al.*, 1984). The creamy/ white transparent, shining, mucoid, smooth and circular colonies was selected as putative Azotobacter isolates traits as presented in Fig.1. Subsequent identification of thirty different isolates was studied based on their cell morphology and Gram's staining reaction under microscope. All the isolates were gram negative with pink coloured and rod to oval shaped cells as shown in Fig.2. Studies of Jimenez *et al.*, 2011 also reported similar colony and cell morphology, signifying that *Azotobacter* is a Gram negative, rod to oval shaped bacteria.

Biochemical characterizations were made of all isolates including the IARI reference strain. Isolates A-2, A-8, A-16, A-23, A-24 and A-28 showed positive results in all the biochemical tests, whereas 24 other isolates showed variable results for different biochemical tests. It indicates that isolates collected from various locations were distinct. Similar results were also reported by Gomare *et al.*, 2013). All the isolates were inhibited by Ciprofloxacin (Cf) and Co-Trimoxazole (Co) as evident by formation of clear zone around the antibiotic disc. The largest clear zone formed by Ciprofloxacin was 15mm. The isolates were less inhibited by other antibiotics used in the studies as evident by formation of smaller clear zone around the antibiotic disc as compared to Cf and Co. (Fig. 3). All the isolates were found resistant to Amoxyclav (Ac), and Erythromycin (E). Similar studies were also conducted by Sindhu *et al.*, 1989.

All the isolates (A-1 to A-30) including IARI reference strain showed good growth at pH 8. At pH 7, isolates A-13, A-17, A-19 and A-20 showed no growth. Results showed that isolates namely A-5, A-7, A-27 A-29 and IARI strain had maximum growth at pH 7. At pH 6, all isolates grew well except A-15, A-19 and A-20. These results showed that the isolates are distinct. Similar studies were also conducted by Jimenez *et al.*, 2011. Keeping the above results in view it will be worthwhile to further characterize the above isolates at molecular level and their efficiency for nitrogen fixation in various field crops.

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