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Isolation, characterization and morphological study of *Azotobacter* isolates

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Abstract: Among the diazotrops, great attention has been paid to the genus *Azotobacter* and its role in increasing the growth and health of plants. In the present study, forty two strains of *Azotobacter* were isolated from soil. These strains were purified and characterized through microscopical and biochemical test for cell shape, pigmentation, colony size, Gram reaction and catalase activity were identified as *Azotobacter* sp These strains showed wide variability to these characters. Among 42 isolates, 7 were single cocci, 7 coccidal chain and 4 were cocci in clumps. Majority of isolates i.e. 24, were small, medium and large rod shaped. Thirty two isolates were Gram –ve, catalase positive and 10 were Gram +ve, catalase negative. Finally from these isolates, twenty two were confirmed as *Azotobacter* strains on cyst formation. The carbon-source utilization pattern revealed that out of 22 strains that 16 strains resembled the characters of *A. chroococcum*, 3 matched with *A. vinelandii* and 3 with *A. beijerinckii.* All 22 isolates were analyzed for its nitrogen fixing ability by using Microkjeldhal method. The highest amount of N_2 (18.88 mg g^{-1} sucrose) was fixed by *Azo*-SBT 72 while lowest (6.04 mg g^{-1} sucrose) by *Azo*-SUR 25 strain. However, injudicious and hazardous use of chemical fertilizers have degraded the soil health and there is need of ecofriendly management of soil by screening and hunting of potential nitrogen fixing strains to protect the soil environment and health. In this context, biofertilizers hunting natural environment is the need of soil to ensure better health of future generations.

Keywords: *Azotobacter* isolates, Catalase activity, Cyst formation

INTRODUCTION

Plant rhizosphere is a very complex niche in which soil, plant and microbe interaction occurs, and plant roots harbours a multitude of different beneficial and deleterious microorganisms. It is due to exudation contains vast organic mix substances such as acids, carbohydrates, nucleic acid derivatives, vitamins, enzymes and other many growth promontory compounds. In this niche, aerobic bacteria belonging to genus *Azotobacter* play a key role in maintaining soil fertility through several beneficial effects in the rhizosphere of cereals and grasses. In addition to biological nitrogen fixation, production of growth stimulates (IAA, gibberellins, cytokinins), inhibition of phytopathogenic fungi through antifungal substances, *Azotobacter* maintains a healthy niche for growing plants (Islam *et al*, 2008).
In hill agriculture, soils have always been rich in biodiversity and prosperous in biomass. In India Uttarakhand is a state where the chemical N fertilization is completely avoided, and organic farming is still in practice. Isolation and characterization of native strains adapted to local environment is extremely important for several reasons. It may contribute to the formulation of an effective bio-inoculants because indigenous strains of rhizobacteria generally posses more competitive ability to survive and influence the growth of inoculated plants (Khalid *et al.*, 2004). It may also provide a chance to reisolate the soil medium and confirm identity with confidence. The present investigation was conducted for isolation, characterization and morphological study of *Azotobacter* isolates.

MATERIALS AND METHODS

Collection and isolation of indigenous *Azotobacter* isolates: Soil samples were collected during October to December, 2006 in different areas of Almora and U.S. Nagar districts in *Kumaun* and *Tarai* regions of Uttarakhand. The samples were collected from rhizospheric soil of different crops (barley, ragi, peas, grass, paddy, amaranth, turmeric and wheat).
For the isolation of different *Azotobacter* spp. on nitrogen free Jensen's medium, sucrose 10 g, dipotas-

sium hydrogen phosphate 1 g, magnesium sulphate 0.5 g, sodium chloride 0.5 g, ferrous sulfate 0.1 g, sodium molybdate 0.005 g, agar 20 g, for 1 liter, pH 7.0-7.2 (Jensen, 1955) was used. Those isolates showing characteristic growth, pigmentation and biochemical reactions of *Azotobacter* species as described in Bergey's Manual of Systemic Bacteriology (Krieg *et al.*1994) purified and classified as *A. vinelandii, A. chroococcum* and *A. beijerinckii*.

Soil samples were collected by taking out rhizosphere soil of different cereal crops along with plant roots. The soil was carefully removed from the plant roots and kept in fresh plastic bags after labelling and tagging. These samples were preserved in refrigerator at 4°C temperature for further use and analyzed for major soil properties as given in Table 1.

The sampled rhizosphere soil was mixed thoroughly to make a composite soil. Then 10 gm of sub-soil sample diluted to 100 ml that considered being 10^{-1} dilution factor. Transferring of 1 ml of 10^{-1} dilution to 9 ml sterilized water with the help of a sterilized pipettes yielded 10^{-2} dilution. In this way, a series of up to 10^{-6} dilution was prepared under aseptic condition.

Glassware such as petriplates, pipettes, beakers, conical flasks, test tubes *etc*. used in the study were sterilized in hot air oven at 160°C temperature for at least 2 hours. Similarly, water, medium were sterilized by autoclaving at 15 pounds per square inch (1.05 kg cm^{-2}) pressure for 30 minutes.

Plating method: To prepare medium plates, approximately 12-15 ml of sterilized, cool and melted (45°C) medium was transferred aseptically into petriplates under laminar flow. After solidification of medium, one ml of suspension from 10^{-4}, 10^{-5} and 10^{-6} dilutions of each rhizosphere soil sample was poured into each medium plate in triplicate. The plates were incubated at 28±2°C in an incubator in inverted position for 7-10 days and growth of presumptive *Azotobacter* was observed on each day during the entire 7 days incubation period. Each well developed colony of *Azotobacter* which differed in morphology, physical appearance, size (3 to 5 mm in diameter or sometimes even more) and colour was picked up and assumed as a distinct strain.

Later on, the morphological and biochemical characteristics were compared with those defined in Bergey's manual (Krieg *et al.*1994) to confirm them as *Azotobacter* strains. The strains with similar characters of *Azotobacter* were streaked onto another medium plate and were purified by subsequent streaking after each growth till all the colonies in petriplates appeared similar in morphology and characters.

Morphological characterization of *Azotobacter* isolates: Colony characteristics such as shape, size and appearance of each strain of *Azotobacter* were recorded as: Cell shape - Oval, rod (small/ medium/ large), oval in chain, rod in chain shaped. Colony shape and size - Raised, flat on the top, domed, conical, regular and irregular. Colour- White, milky white, watery, mucoid and viscous. Consistency- Based on light transmitted through the colony, these were categorized as transparent, translucent and opaque colonies (usually firm with little gum whereas the less dense colonies were often gummy, soft, glistening or dull).

All isolates of *Azotobacter* were subjected to react with Gram stains as per standard staining procedure described by Cerney (1993).

Diluted bacterial cell suspension was taken with the help of an inoculating loop and it was uniformly spread over in the centre of a clean microscopic slide. The bacterial cells were fixed by heating it through passing repeatedly few seconds over a flame. They were stained with a drop of crystal violet solution (30 seconds), iodine (30 seconds) followed by ethyl alcohol and safranin (1 to 1.5 minutes) solution. After each staining period, the cells were washed under tap water jet and examined under compound microscope using oil immersion lens and these bacterial cells were classified as Gram negative (G-) or gram positive (G+) or gram variable (G±) strains.

The pigment of all colonies of *Azotobacter* isolates was noted after a week on a Jensen's agar medium. All isolates of *Azotobacter* were subjected to react with Violamine stain (Winogradsky, 1949) for 45 sec, than washed and counterstained with dilute crystal violet (0.05%) for 1 minute. After rinsing with water, the preparation was mounted in water. To investigate the cyst forming ability of *Azotobacter* and to observe the ultra-structure of *Azotobacter* cells, electron-microscopy was carried out. Those cells that formed cysts confirmed as *Azotobacter* and rest those not formed cysts were considered as *Azomonas.*

The biochemical test was performed as catalase activity test. The test shows the ability of some microbes to degrade hydrogen peroxide by producing the enzyme catalase. Usually,

all *Azotobacter* species have the capacity to produce oxidases and catalases for the protection of their nitrogenise (Jimenez, *et al.* 2011). About 1 ml of 3 % of H_2O_2 was poured over *Azotobacter* culture on microscopic slide or in broth. Intensive bubbling (*Azotobacter*) was noted as catalase positive, slight bubbling was weakly positive; no gas production was catalase negative.

Identification and nomenclature of *Azotobacter* strains: Each isolated *Azotobacter* strain was named by suffixing *Azo* by three alphabetical letters followed by a number as procedure given below in Table 2. The first letter denotes the first letter of the name of the researcher, middle one denotes the place of its origin, the last letter denotes the rhizospheric crop plant from which it was isolated followed by a number at the end, indicating the strain number which differed with others in cultural/ morphological/ biochemical characteristics. Based on cultural, morphological and biochemical characters all colonies are classified as shown below in Table 3, the confirmation (authenticity) of being genus

Table 1. Characteristics of sampling sites used for isolation of *Azotobacter* strains.

Properties	Locations			
	Pantnagar, U.S. Nagar			Almora
	Beni	Phoolbagh	*NECRC	Udaipur
Sand (%)	8.84	38.5	42.3	33.8
Silt (%)	61.41	30.8	30.0	43.4
Clay (%)	29.75	30.7	27.7	22.8
Soil texture	Silty clayloam	Clay loam	Clay loam	Loam
pH	7.30	7.10	6.15	6.88
O.M. (%)	1.31	1.36	1.26	1.39
Available P_2O_5 (kg ha^{-1})	14.2	10.6	14.8	12.8
Available K_2O (kg ha^{-1})	196	188	176	164
Coordinates	29° 00' 56" N 79° 30' 7.8"E	28° 01' 29" N 79° 28' 20" E	29° 01' 6" N 79° 28' 40"E	29° 50' 53" N 79° 11' 37" E

*NECRC = Norman E. Borlog Crop Research Centre, Pantnagar, U.S. Nagar

Table 2. Nomenclature of isolated *Azotobacter* strains.

S.N.	Sampling site	Rhizosphere	Strain name	Symbols of presumptive *Azotobacter* strains
1	Beni, Pantnagar (B)	Barley (B)	*SBB	SBB 1
2	Udaipur, Almora (U)	Ragi (R)	SUR	SUR 2, SUR 21, SUR 22, SUR 23 etc.
3	Phoolbag, Pantnagar (P)	Peas (P)	SPP	SPP 3, SPP 31, SPP 32
4	Phoolbag, Pantnagar (P)	Grass (G)	SPG	SPG 4, SPG 41, SPG 42
5	CRC, Pantnagar (C)	Rice (R)	SCR	SCR 5, SCR 51, SCR 52 etc
6	Udaipur, Almora (U)	Amaranth (A)	SUA	SUA 61, SUA 62, SUA 63 etc.
7	Beni, Pantnagar (B)	Turmeric (T)	SBT	SBT 7, SBT 71, SBT 72 etc.
8	Udaipur, Almora (U)	Wheat (W)	SUW	SUW 8, SUW 81, SUW 83 etc.

*S = Sandeep (*Initial letter = Name of the researcher); B = Beni; B = Barley crop (collectively SBB)

Azotobacter had been made as per the description given in Bergey's Manual of Systemic Bacteriology (Holt and Krieg, 1984). Aquilanti *et al.* (2004) described different strategies to isolate *Azotobacter* from soil samples. He concluded that soil pasteplate method combined with isolation of mannitol agar proved to be the best strategy in terms of reliability and selectivity.

Determination of dinitrogen fixing capacity: The nitrogen fixing efficacy of each strain was determined in terms of the amount of total nitrogen fixed in 25 ml broth culture in 7 days under bacteriological controlled conditions shown below in Table 5. Jensen's broth containing all the ingredients, except agar was prepared in Jensen (1955). After pouring 25 ml broth in each 150 ml conical flask and plugging with cotton wool, the flasks were autoclaved. After cooling, each flask was inoculated by adding 0.1 ml broth culture of the each strain in triplicate and the flasks were shaken on a rotary shaker for 7 days at 28 ± 2˚C. From each flask, 5 ml broth culture was used to determine the total nitrogen by the microkjeldahl method as described by Jackson (1967). Further, the population of *Azotobacter* in each broth culture was determined by serial dilution pour plate method.

The total number of *Azotobacter* cells per gram soil was calculated by multiplying the average number of colonies with 10^5 as dilution factor, using the following formula.

No. of *Azotobacter* cells in broth culture (c.f.u.ml^{-1}) = No. of colonies $plate^{-1}$ × dilution factor

RESULTS AND DISCUSSION

Characterization of *Azotobacter* isolates

Cell / colony shape and size: The morphological characters of *Azotobacter* isolates revealed that cells of *Azotobacter* isolates namely *Azo*-SUR 2, *Azo*-SPP 3, *Azo*-SUA 63, *Azo*-SUA 68, *Azo*-SUA 69, *Azo*-SUW 83, *Azo*-SUW 84 were cocci in single, while the cells of *Azo* SUR 21, *Azo*-SPG 4, *Azo*-SCR 5, *Azo*-SCR 51, *Azo*-SCR 53, *Azo*-SUA 66, *Azo*-SUA 67 were cocci in chains and cells of *Azotobacter* isolates *Azo*-SPG 41, *Azo*-SCR 54, *Azo*-SCR 56, and *Azo*-SBT 74 were cocci in clumps.

Remaining other strains, *Azo*-SBB 1, *Azo*-SUA 6, *Azo*-SUA 62, *Azo*-SUA 64 were single rods, while *Azo*-SUR 22, *Azo*-SPP 31, *Azo*-SPG 42, *Azo*-SCR 55, *Azo*-SUA 61, *Azo*-SUA 65, *Azo*-SUW 8, were rods in chain. The cells of *Azo*-SUR 25, *Azo*-SPP 32 were small rods. The cells of *Azo*-SUR 23, *Azo*-SUR 26, *Azo* -SPP 33, *Azo*-SCR 52, *Azo*-SBT 7, *Azo*-SBT 72, *Azo*-SBT 73 isolates were medium rods, however, long rods were observed in *Azo*-SUR 24, *Azo*-SBT 71, *Azo*-SUW 81 and *Azo*-SUW 82 isolates. The morphological characters of *Azotobacter* isolates showed that among 42 isolates, 7 were single cocci, 7 coccidal chain and 4 were cocci in clumps. Majority of isolates i.e. 24, were small, medium and large rod shaped/bacilli (Table 3).

The colonies developed on Jensen's agar medium were raised, spherical flat and few with irregular margins. The colony size varied from ≥2 mm to ≥5 mm in 7 days. It is evident that *Azotobacter* isolates were pleo-

Table 3: Morphological and cultural characteristics of isolated *Azotobacter* strains (Colony characteristics one week after streaking).

S. N.	*Azotobacter* name	Cell shape	Colony shape / margin	Size (mm)	Colour	Consistency	Gram staining	Catalase test
1	*Azo*-SBB1	Rod singly	Spherical, punctiform	2.8	Dull white	Slimy	-	+
2	*Azo*-SUR 2	Oval	Spherical, flat	3.8	Dull white	Mucoid	-	+
3	Azo-SUR 21	Oval in chains	Circular, smooth	3.0	Dull white to brownish Clear	Viscous	-	+
4	*Azo*-SUR 22	Rod in chains	Circular, raised	4.2	Clear white	Milky	-	+
5	*Azo*-SUR 23	Medium rod	Circular, raised	4.2	Pale yellow	Mucoid	-	+
6	*Azo*-SUR 24	Large rod	Circular, smooth, pulvinate	5.6	Dull white	Viscous	-	+
7	*Azo*-SUR 25	Small rod	Rhizoid, flat	2.8	Creamy white, off-white	Dry-Diffused	+	-
8	*Azo*-SUR 26	Medium rod	Spherical, convex	5.0	White to light yellow	Slimy	-	+
9	*Azo*-SPP 3	Spherical	Large, slimy, glisten-ing	3.0	Dull white	Glistening, Gummy	-	+
10	*Azo*-SPP 31	Rod in chains	Smooth, circular and highly convex	2.5	Dull white to yellowish	Mucoid, viscid	-	+
11	*Azo*-SPP 32	Small rod	Irregular, large	2.8	Un-pigmented to whitish	Slimy	-	+
12	*Azo*-SPP 33	Medium rod	Rhizoid, flat	2.9	Clear	Dry	-	+
13	*Azo*-SPG 4	Cocci in chains	Filamentous	2.9	Pale yellow	Dry	-	+
14	*Azo*-SPG 41	Cocci in clumps	Circular smooth	3.0	Pale yellow	Dry	-	+
15	*Azo*-SPG 42	Rod in chains	Circular, flat margin	3.2	White	Weak slimy	-	+
16	*Azo*-SCR 5	Cocci in chains	Rhizoid Irregular, radiate	2.8	Pale yellow	Dry	+	-
17	*Azo*-SCR 51	Cocci in chains	Spherical, flat	2.9	Dull white	Milky	+	-
18	*Azo*-SCR 52	Cocci in clumps	Slimy, circular and convex	2.8	Pale yellow to Brown	Milky	-	+
19	*Azo*-SCR 53	Cocci in chains	Slimy, circular, con-vex	2.0	White to yellow brown	Milky	-	+
20	*Azo*-SCR 54	Cocci in clumps	Entire, circular, raised	2.3	Dull white to brown	Viscous	-	+
21	*Azo*-SCR 55	Rods in chains	Radiate, raised	2.3	Creamy white to brown	Slimy	-	+
22	*Azo*-SCR 56	Cocci in clumps	Poor growth	2.8	White to black brown	Viscous	+	-
23	*Azo*-SUA 6	Rod singly	Poor growth	3.0	Yellowish to white	Viscous	+	-
24	*Azo*-SUA 61	Rods in chains	Slimy, circular and convex	3.3	Clear translucent	Mucoid	+	-
25	*Azo*-SUA 62	Rods singly	Spherical, Punctiform	2.8	Yellowish to brown	Milky	-	+
26	*Azo*-SUA 63	Cocci singly	Slimy, circular and convex	3.5	Dull white	Viscous	-	+
27	*Azo*-SUA 64	Rods singly	Spreading, convex	4.5	Brownish	Viscous	-	+
28	*Azo*-SUA 65	Rods in chains	Circular, contoured, convex	3.0	Yellowish white	Mucoid	-	+
29	*Azo*-SUA 66	Cocci in chains	Spherical, Punctiform margin	3.0	Yellowish to white	Viscous	-	+
30	*Azo*-SUA 67	Cocci in chains	Poor growth	2.5	Yellowish to white	Dry	+	-
31	*Azo*-SUA 68	Cocci single	Poor growth	2.8	Yellowish	Dry	+	-
32	*Azo*-SUA 69	Cocci single	Poor growth	2.0	Clear	Dry	+	-
33	*Azo*-SBT 7	Medium rod	Slimy, circular and convex	4.2	Yellowish to white	Viscous	-	+
34	*Azo*-SBT 71	Large single rod	Entire, convex and spreading	5.6	Brown	Viscous	-	+
35	*Azo*-SBT 72	Medium rod	Slimy, circular and convex	4.0	Brownish black	Milky	-	+
36	*Azo*-SBT 73	Medium rod	Slimy, circular and convex	4.5	Dull white to brownish black	Slimy	-	+
37	*Azo*-SBT 74	Cocci in clumps	Poor growth	3.3	Clear	Milky	+	-
38	*Azo*-SUW 8	Rods in chains	Slimy, circular and convex	4.2	White to brownish black	Milky	-	+
39	*Azo*-SUW 81	Large rods	Circular, wrinkled	4.5	Creamy white	Gummy	-	+
40	*Azo*-SUW 82	Large rods	Spreading, convex	4.0	Yellowish brown-black	Mucoid	-	+
41	*Azo*-SUW 83	Cocci single	Spherical, punctiform	3.6	Dull Brown	Viscous	-	+
42	*Azo*-SUW 84	Cocci single	Wrinkled, raised	3.2	Clear	Milky	-	+

Table 4: Discrimination of *Azotobacter* isolates characters for classification into different species.

S. N.	*Azotobacter* strains	Carbon source utilized			Cyst	Extracellular polysaccharide production	Result
		Starch	Maltose	Rhamnose			
1	*Azo*-SUR 2	-	-	-	+	+	*A. beijerinckii*
2	*Azo*-SUR 21	-	-	-	+	+	*A. beijerinckii*
3	*Azo*-SUR 22	+	+	-	+	+	*A. chroococcum*
4	*Azo*-SUR 23	+	+	-	+	+	*A. chroococcum*
5	*Azo*-SUR 24	+	+	-	+	+	*A. chroococcum*
6	*Azo*-SUR 25	+	+	-	+	+	*A. chroococcum*
7	*Azo*-SUR 26	+	+	-	+	+	*A. chroococcum*
8	*Azo*-SPP 3	+	+	-	+	+	*A. chroococcum*
9	*Azo*-SPP 31	+	+	-	+	+	*A. chroococcum*
10	*Azo*-SPG 42	+	+	-	+	+	*A. chroococcum*
11	*Azo*-SCR 52	-	+	+	+	+	*A. vinelandii*
12	*Azo*-SCR 53	+	+	-	+	+	*A. chroococcum*
13	*Azo*-SCR 55	+	+	-	+	+	*A. chroococcum*
14	*Azo*-SUA 61	+	+	-	+	+	*A. chroococcum*
15	*Azo*-SUA 63	+	+	-	+	+	*A. chroococcum*
16	*Azo*-SUA 65	+	+	-	+	+	*A. chroococcum*
17	*Azo*-SBT 7	-	+	+	+	+	*A. vinelandii*
18	*Azo*-SBT 71	-	+	+	+	+	*A. vinelandii*
19	*Azo*-SBT 72	+	+	-	+	+	*A. chroococcum*
20	*Azo*-SBT 73	+	+	-	+	+	*A. chroococcum*
21	*Azo*-SUW 8	+	+	-	+	+	*A. chroococcum*
22	*Azo*-SUW 82	-	-	-	+	+	*A. beijerinckii*

morphic in nature. The variation in cell shape, colony size and its appearance was because of various factors such as presence of disorganized, shapeless masses-symplasms, giant spindle shaped cells branched form and even spore formation in cultures of *Azotobacter* (Pleomorphism) may also be attributed to the composition of the medium and the stage of its growth in the medium as has been reported by Aquilanti *et al.* (2004). The colony characters such as colony-margin, size, colour and consistency also differed among different isolates of *Azotobacter* (Table 4). Thirty two isolates were Gram -ve and 10 were Gram +ve, hence were not *Azotobacter* isolates. It is in agreement with the obtained results by (Tejera, *et al.* 2005; Ahmad, *et al.* 2008).

Gram staining test, catalase activity test: Thirty one *Azotobacter* isolates were found catalase positive while 11 isolates were catalase negative (Table 4) hence were not *Azotobacter*. The isolates of *Azotobacter* were studied for their carbon source utilization and cyst forming behaviour to confirm and differentiate them into different species according to the Bergey's Manual of Systematic Bacteriology (Table 4). Thirty one isolates showed catalase positive test and eleven catalase negative.

Colony colour and consistency: Different *Azotobacter* isolates showed wide variability in consistency and colony colour. Isolates were reported unevenness in colour and flowing consistency mucoid, viscous, milky, glistening, gummy with dull to cream white, pale yellow, brown as described by John *et al.* (1994). *Azo*-SBT 7, *Azo*-SBT 72, *Azo*-SBT 73 isolates were medium rods, however, long rods were observed in *Azo*-SUR 24, *Azo*-SBT 71, *Azo*-SUW 81 and *Azo*-SUW 82 isolates.

Carbon source utilization and cyst behaviour: It is evident from the carbon source utilization behaviour of 22 strains that 16 strains resembled the characters of *A. chroococcum*, 3 matched with *A. vinelandii* and 3 with *A. beijerinckii.* This confers the reports that *A. chroococcum* is the most commonly encountered species among the genus *Azotobacter* (Voets and Dedeken, 1966) also survival of *A. chroococcum* in Nainital *tarai* as reported by Banerjee (1977).

The most important character of *Azotobacter* is its ability to fix atmospheric nitrogen by the enzyme popularly known as nitrogenase. The nitrogen fixation by *Azotobacter* has been reported from 2-15 mg N g^{-1} of carbon utilized (Becking, 1992). however Ravikumar *et al.* (2004), Stella and Suhaimi (2010) also reported these three species of *Azotobacter*, *A. chroococcum*, *A. vinelandii* and *A. beijerinckii*, exhibited high growth, nitrogen fixation and in vitro production of phytohormone.

***Azotobacter* population and their N_2-fixing efficiency in broth:** The viable counts of all 22 *Azotobacter* strains varied from 23 – 89 × 10^6 c.f.u. ml^{-1} broth. Highest population of 89.32 × 10^6 c.f.u. ml^{-1} broth was recorded with strain *Azo*-SUR 23, however, strains *Azo* -SBT 7, *Azo*-SBT 72, *Azo*-SBT 73 and *Azo*-SUW 82 were at par with strain *Azo*-SUR 23. The minimum number of cells 23 × 10^6 c.f.u. ml^{-1} broth was recorded in *Azo*-SUA 61 strain.

The amount of N_2 fixed by different strains ranged

Table 5. Population of *Azotobacter* strains in broth and amount of N_2 fixed by them.

S. N.	*Azotobacter* Strains	Population ($\times 10^6$ c.f.u. ml^{-1})	N_2 Fixed (mg g^{-1} Sucrose)
1	Control	--	--
2	*Azo*-SUR 2	62.33	11.04
3	*Azo*-SUR 21	66.05	17.40
4	*Azo*-SUR 22	70.93	9.09
5	*Azo*-SUR 23	89.32	12.08
6	*Azo*-SUR 24	39.71	7.00
7	*Azo*-SUR 25	45.35	6.04
8	*Azo*-SUR 26	58.99	9.21
9	*Azo*-SPP 3	55.35	9.80
10	*Azo*-SPP 31	28.08	10.34
11	*Azo*-SPG 42	69.80	10.10
12	*Azo*-SCR 52	56.97	12.34
13	*Azo*-SCR 53	65.45	11.44
14	*Azo*-SCR 55	24.08	10.72
15	*Azo*-SUA 61	23.00	6.12
16	*Azo*-SUA 63	35.81	13.28
17	*Azo*-SUA 65	44.75	16.23
18	*Azo*-SBT 7	83.38	10.42
19	*Azo*-SBT 71	66.16	16.85
20	*Azo*-SBT 72	83.92	18.88
21	*Azo*-SBT 73	87.75	16.92
22	*Azo*-SUW 8	72.66	9.66
23	*Azo*-SUW 82	85.32	18.42
	SEm±	2.98	1.29
	CD at 5%	8.50	3.62
	CV	8.63	4.48

from 6.04 to 18.88 mg g^{-1} sucrose. Highest amount of nitrogen was fixed by *Azo*-SBT 72 while lowest by *Azo*-SUR 25. The strains namely *Azo*-SUR 21, *Azo*-SUA 65, *Azo*-SBT 71, *Azo*-SBT 73 and *Azo*-SUW 82 did not differ significantly from *Azo*-SBT 72. Other strains fixed significantly lower nitrogen than *Azo*-SBT 72. The strain *Azo*-SBT 72 had 3.12 fold N_2 fixation power over *Azo*-SUR 25. All the *Azotobacter* strains fixed significantly more N_2 except *Azo*-SUR 22, *Azo*-SUR 24, *Azo*-SUR 26, *Azo*-SUA 61 and *Azo*-SUW 8 than *Azo*-SUR 25 and *Azo*-SPP 3. Yoshida (1972) also reported that biological nitrogen fixation in Philippine rice fields were ranged from 2.30 to 33.3 kg ha^{-1}. Similarly results were obtained by Narayan and Kehri (2011) studying effect of different agricultural practices on population dynamics of *Azotobacter chroococcum* and its nitrogen fixing potentiality in Trans-Ganga and Trans-Yamuna plains of India.

All the *Azotobacter* strains differed in their viable counts in broth culture. The number of cells depends upon the generation time and physiological characteristics of the strains, therefore, the population varied in the broth. This is well known fact in conformity with Brock *et al*. (1994). The N_2 fixing capacity of *Azotobacter* strains also differed from strain to strain. The fixation of N_2 depends upon the activation of nitrogenase enzyme, which may vary from strain to strain. The wide variations in nitrogen fixing capacity of different strains of *Azotobacter* have also been reported by Sanoria and Sundara Rao (1975), Mahmoud *et al.* (1978) and Tippannavar and Reddy (1987). These isolation results have close conformity with findings of Torres, *et al.* (2000). They obtained *Azotobacter chroococcum*, *A. vinelandii*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia* strains from rhizosphere of rice cultivated in the Tolima region,

Conclusion

Based on the performed study of isolation, characterization of *Azotobacter* it was found that some *Azotobacter* species namely *A. chroococcum*, *A. vinelandii* and *A. beijerinckii* were well distributed in crop growing soils of *Tarai* and *Kumaun* region. The bacteria showed Gram negative, catalase positive, cyst forming nature though, pleomorphism in shape. On carbon source utilization behaviour, *Azotobacter* showed its species variation as out of 22 strains that 16 strains confirmed the characters of *A. chroococcum*, 3 matched with *A. vinelandii* and 3 with *A. beijerinckii.* Among these isolates peak amount of N_2 fixed was 18.88 mg g^{-1} sucrose by a strain *Azo*-SBT 72. Therefore, the genus *Azotobacter* was active in the maintenance of soil fertility and productivity of local farms. Isolation, purification and screening out highly efficient *Azotobacter* strains from natural field showed potentiality to find out better inoculants preparation for further research trials.

REFERENCES

Ahmad, F, Ahmad, I. and Khan, M.S. (2008). Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. *J. Microbiol. Research.* 163: 173-181.

Aquilanti, L., Favilli, F. and Clementi, F. (2004). Comparison of different strategies for isolation and preliminary identification of *Azotobacter* from soil samples. *Soil Biol. Biochem.*, 36: 1475-1483.

Banerjee, S. (1977). Morphological and physiological characteristics of different strains of *Azotobacter* isolated from soils of Nainital *tarai*. *Pantnagar J. Res.*, **2**(2): 262.

Becking, J.H. (1992). The family *Azobacteraceae*, 2nd ed, Springer, Verlag, Berlin, pp. 3144–3170.

Brock, T.D., Madigan, T.M., Martinko, J.M. and Parker, J. (1994). Biology of Microorganisms. 7th ed. Prentice Hall International, Inc., USA. p. 142.

Cerney, G. (1993). Method for the distinction of gram negative and gram positive bacteria. *EU J. Appl Micro*. (3), 223-225.

Holt, J.G. and Krieg, N.R. (ed.) (1984). Bergey's Manual of Systematic Bacteriology, vol. 1. Wiliams and Wilkins, Baltimore, Md, pp 219.

Islam, M.Z., Sharif, D.I. and Hossain, M.A. (2008). A comparative study of *Azotobacter* spp. from different soil samples. *J. Soil Nature*. 2(3): 16-19.

Jackson, M.L. (1967). Soil chemical analysis. Prentice Hall of India Pvt. Ltd., New Delhi. pp. 263-293.

Jensen, H.L. (1955). Non-symbiotic nitrogen fixation *In:* Soil nitrogen (eds.) Barthdlomew, W.V. and Clark, F. E. *American Soc. Agron. Inc. Madison,* 10: 436-480.

Jimenez, J.D., Montana, J.S., Martínez, M.M. (2011). Characterization of free nitrogen fixing bacteria of the genus *Azotobacter* in organic vegetable-grown Colombian soils. *Braz. J. Microbiol.* 42(3) 66-78.

John, G.H., Noel, R.K., Peter, H.A.S., James, T.S. and Stanley, T.W. (1994). Aerobic/ microaerophilic, motile, helical/vibroid Gram negative bacteria. In: Bergey's Manual of Determinative Bacteriology. p. 39–45. USA: Lippincott Williams & Wilkins.

Khalid, A., Arshad, M. and Zaheer, Z.A. (2004). Screening plant promoting rhizobacteria for improving growth and yield of wheat. *J. Appl. Microbiol.*, 96: 473-480.

Krieg N.R., Holt J.G., Sneath P.H.A., Staley J.T., and Williams S.T. (1994). Bergey's Manual of Determinative Bacteriology (9th ed.), Williams & Wilkins, Baltimore, Md, USA.

Mahmoud, S.A.Z., El-Sawy, M., Ishac, Y.Z. and El-Safty, M.M. (1978). The effects of salinity and alkalinity on the distribution and capacity of N_2 fixation by *Azotobacter* in Egyptian soils. *Ecol. Bull. NFR* (*Naturvetensk. Forskningsradet*) 26: 99-109.

Narayan, R.P. and Kehri, H.K. (2011). Effect of different agricultural practices on population dynamics of *Azotobacter chroococcum* and its nitrogen fixing potentiality in Trans- Ganga and Trans-Yamuna plains of India. *Acta Agriculture Serbia*, 16.97-122.

Ravikumar, S., Kathiresan, K., Ignatiammal, S.T.M., Selvam, M.B. and Shanty, S. (2004). Nitrogen-fixing *azotobacters* from mangrove habitat and their utility as marine biofertilizers. *Journal of Experimental Marine Biology and Ecology* 312: 5–17.

Sanoria, C.L. and Sundara Rao, W.V.B. (1975). Effect of seed bacterization with *Azotobacter chroococcum* on sorghum and wheat. *Indian J. Agric. Sci*. 45: 224-226.

Stella, M. and Suhaimi, M. (2010). Selection of suitable growth medium for free-living diazotrophs isolated from compost. *J. Trop. Agric. and Fd. Sc.* 38(2): 211–219.

Tejera, N., Luch, C., Martinez-Toledo, M.V., Gonzalez-Lopez, J. (2005). Isolation and characterization of *Azotobacter* and *Azospirillum*strains fromsugarcane rhizosphere. *J. Plant and Soil*: 223–232.

Tippannavar, C.M. and Reddy, T.K.R. (1987). Population dynamics of *Azotobacter* in different soil types, their nitrogen fixing capacities and effect of *Azotobacter* isolates on wheat seed germination. *J. Farm. Sys.* 3(3-4): 67-71.

Torres-Rubio, M.G., Valencia-Plata, S.A., Bernal-Castillo J. and Martienez-Nieto, P. (2000). Isolation of Enterobacteria, *Azotobacter sp.* and *Pseudomonas sp.*, Producers of Indole-3-Acetic Acid and Siderophores from Colombian Rice Rhizosphere. *R.L. De Microbiologiea*, 42: 171-176.

Voets, J.P. and Dedeken, M. (1966). A physiological approach to the classification of the genus *Azotobacter* (c.f.) The biological fixation of atmospheric nitrogen by free living bacteria. *In* : "Soil Biology: Review of Research". *Nat. Resour. Res. UNESCO*, 9: 65-125.

Winogradsky, S.N. (1949). Microbiologie du soil problems et methods (e.f.). The *Azotobacteraceae*. *In*: *Bacterial Review*. 18: 199.