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Variability in *Azotobacter* pertaining to physio-morphological characteristics and nitrogen fixing ability

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Abstract
The current investigation was carried out with the aim to study the variability in *Azotobacter* species pertaining to physio-morphological characteristics and nitrogen fixing ability. It revealed that, among 50 different isolates obtained from the soil samples of Maharashtra, 11 isolates belonged to *A. beijerinckii* and remaining 39 isolates belonged to *A. paspali*. The isolates PN 7, PN 8, AH 1 (*A. paspali*) and PN 2 (*A. beijerinckii*) were found to be fixing nitrogen greater than 30 mg / g of sucrose, whereas the isolates ST 6, AH 6, AH 18, AH 7, AH 9, AH 17 (*A. paspali*) and AH 20 (*A. beijerinckii*) were fixing atmospheric nitrogen lesser than 10 mg / g of sucrose consumed. Both the species *Azotobacter beijerinckii* and *Azotobacter paspali* had shown similar results in Gram staining reaction, catalase and oxidase test, indole formation, gelatin liquefaction, carbon source utilization test but showed variation in morphological, cultural characteristics, motility test, starch hydrolysis test and nitrogen fixing efficiency.

Keywords: *Azotobacter*, morphological, biochemical characteristics, nitrogen fixing ability

1. Introduction
The technology development in recent years had intervened agriculture and soil wealth to a greater extent, especially the usage of synthetic fertilizers. Even though it offers a great advantage of supplying the essential nutrients in the readily available form to the plants, they pose a problem of affecting the plant rhizospheric organisms and soil health. (Kaviyarasan *et al.*, 2020) [8]. Hence, biofertilizers are an alternate choice and one among the popular biotechnology inventions, which is essential to support and enhance development of sustainable agriculture, green, non-pollution agriculture and organic farming. Nitrogen fixing organisms is the vital group of biofertilizers.

Among the nitrogen fixing bacteria, the genus *Azotobacter* is a well-known organism for nitrogen fixation in the soil by forming ammonium (NH^{4+}) ions. (Agarwal *et al.*, 2018) [1]. Besides nitrogen fixation, it is also involved in production of several plant growth promoting substances (Indole Acetic Acid (IAA), Gibberellins (GA_3), Auxins and Cytokinins) that performs various plant functions *viz.*, cell division, seed germination, flowering, seed dormancy and protection against deleterious pathogens. (Lhamo *et al.*, 2022) [9]. *Azotobacter* population is generally low in the uncultivated soils and around the plant rhizosphere (Wani *et al.*, 2013) [17]. It can be isolated from the plant roots of several crops including cereals, vegetables and plantation crops (Arun, 2007) [2].

Azotobacter, a free living heterotrophic organism, belongs to the family Azotobacteriaceae (Becking, 1981) [3]. Being a nitrogen fixer, *Azotobacter* can fix 10 mg of N per g of sucrose (or any carbon source) under *invitro* conditions (Wani *et al.*, 2013) [17]. It also possesses antifungal activity and produces some antibiotics (Vikhe, 2014). It generally grows in the pH range of 4.8-8.5 (Jain *et al.*, 2021) [7] and are sensitive to high temperature (>35 ºC), acidic pH condition and high salt concentrations. *Azotobacter* has 7 different species *viz*, *Azotobacter chroococcum*, *A. armenicus*, *A. paspali*, *A. beijerinckii*, *A. salinestris*, *A. nigricans* and, *A. vinelandii* (Nongthombam *et al.*, 2021) [11].

Azotobacter increases the availability of several nutrients to the plants, stimulate enzymes and essential compounds production, increases the uptake of nutrients, immobilizes the heavy metals present in the soil and exerts changes in growth and productivity of the crops. (Nongthombam *et al.*, 2021) [11]. Soil has the abundant presence of *Azotobacter*, which influences the microbiological properties and soil physico-chemical properties like pH, temperature, soil organic matter and moisture etc. (Hamilton *et al.*, 2011) [5].

Azotobacter possesses enormous plant growth promoting characters by its nature, hence, it becomes necessary to multiply it and produce the biofertilizer for sustainable agriculture and increased yield of the crops. Although, several investigations have been done regarding *Azotobacter* spp. so far, variability in characteristics of *Azotobacter* spp. from various plant rhizospheres of different parts of Maharashtra is found to be an interesting topic. Hence, investigations was made in the *Azotobacter* isolated from the rhizospheres of several crops and they were confirmed by several biochemical tests and nitrogen fixing ability by micro kjeldahl method.

2. Materials and Methods

2.1 Collection of soil samples: The healthy and vigorously growing plants (cereals/ sugarcane/ vegetables) were uprooted from the field and soil adhering to the roots and around the root zone of the plant was collected in the clean disinfected polythene bags. The bags were labelled with the required information (date of collection, crop from which the soil was collected and, name of the village, tehsil and district from where it was collected). The soil samples were collected from different crops and villages belonging to Pune, Satara, Solapur, Ahmednagar and Sangli districts of Maharashtra State. The collected soil samples were coded, where the letters indicated name of the district and figures indicated number given to the sample. Names of the districts were abbreviated as PN for Pune, ST for Satara, SL for Solapur, AH for Ahmednagar and SL for Sangli. For example, Pune district soil samples were coded as PN1, PN2, PN3 etc. The samples were brought to the laboratory and were stored in the refrigerator at a temperature of 4 ºC.

2.2 Isolation of *Azotobacter* spp. from soil sample

Isolation of *Azotobacter* was done using the serial dilution and pour plate technique. Firstly, 1 g of soil sample was weighed and added to the test tubes containing 9 ml sterile water blank, which gave a dilution of the value of 10^{-1}. 1ml of the aliquot from 10^{-1} dilution was transferred with the help of sterile micropipette to the next test tube containing 9 ml sterile water blank, that gave 10^{-2} dilution. Following the same procedure, up to 10^{-6} dilutions were prepared. From 10^{-6} dilution, 1ml of aliquot was taken using the micropipette and transferred to the sterilized Petri plate, after which 10-15 ml of sterilized Jensen's medium was poured in the plates and the plates were rotated in clockwise and anticlockwise direction for few minutes for uniform mixing of the aliquot with the medium. The entire procedure was done under the aseptic condition in laminar air flow cabinet. After solidification of the poured medium, the Petri plates were incubated in an inverted position for 5-7 days in bacteriological incubator at 28 ± 2 ºC temperature. (Upadhyay *et al.* 2015) [15].

2.3 Purification and maintenance of the isolates

After appearance of growth of the colonies on Petri plates, colonies showing distinct colony characters were selected and sub-cultured several times to obtain pure cultures of the bacterial isolates. The well isolated colonies showing distinct colony characters were selected and these were picked using sterile needle and the inoculum was streaked on the Jensen's media slants and kept for incubation for 5-7 days in bacteriological incubator at 28±2 ºC temperature. Later, the slants stored in refrigerator at 4 ºC for further study (Sivapriya *et al.* 2017) [13].

2.4 Morphological characterization of *Azotobacter* isolates

After the incubation of the aliquots inoculated Petri plates for 2-4 days, the bacterial growth was observed in the range of small to moderate colonies on the surface of the medium. Each sample showed variation in the growth of the colonies and were considered as a criteria for morphological characterization of the bacterial isolates, which included the colony shape, size, form, elevation, margin, color, consistency and appearance. (Hala *et al.* 2019) [4].

2.5 Pigmentation of the isolates

The bacterial isolates shows variation in the pigments produced and the characteristic pigmentation of them will be observed. (Sivapriya *et al.* 2017) [13]. *Azotobacter* species produces various colored pigments *viz.*, dull white, creamy white, pale yellow, brown, dark brown and such pigmentation of the isolates was recorded.

2.6 Gram staining

A very thin smear of the pure culture was made on a separate glass slide, followed by air drying for few minutes and it was heat fixed by passing the slide 3-4 times through flame of the spirit lamp. The bacterial smear was then stained with a few drops of crystal violet and it was allowed to stand for 1 minute and then washed with distilled water for few seconds. The slide was then air dried and after drying, the smear was treated with iodine solution for 30 seconds and then decolorized with 95% ethyl alcohol, followed by washing with distilled water and air dried. Later, the smear was counterstained with safranin for 30 seconds and it was washed with distilled water. The slide was blot dried, air dried and the cellular morphology (rod, round, oval) and its arrangements (single, pair, clusters, clumps) was observed under the light microscope.

2.7 Motility test

A pure bacterial culture was picked up using a sterile needle and stabbed it on the motility medium to within 1 cm of the bottom of the tube. Care was taken to remove the needle in the same line or direction it was entered. The stabbed culture medium was incubated at 35°C for 18 hours or until the period of evident bacterial growth. A positive motility result was indicated by a red turbid area extending away from the line of inoculation, whereas negative test was observed by the presence of red growth along the inoculation line and no further development of extension of turbid area.

2.8 Biochemical characterization of the isolates

2.8.1 Catalase test (Smibert and Krieg, 1981) [14].

A loopful of the pure culture was placed over the clean microscopic slide, over which 1 ml of 3% H_2O_2 was poured. Within a few minutes, reaction was observed as intensive bubbles formation indicating that the isolate was catalase positive. On the other hand, the isolates with slight bubbling were indicated as weak positive and no gas or bubbles production was categorized as catalase negative.

2.8.2 Oxidase test

The filter paper was made into pieces and soaked in 1% aqueous tetramethyl-p-phenylenediamine-dihydrochloride. The test culture was scraped with a glass rod and rubbed on the soaked filter paper. After 10-30 seconds, changes were observed. The development of deep violet color on the filter

paper indicated that the culture was oxidase positive and no color formation in the filter paper showed that the organism was oxidase negative.

2.8.3 Indole formation test
Firstly, tryptophan broth was prepared, after which the bacterial isolate was inoculated into the broth and was kept for incubation at a temperature of 37 ºC for a duration of 24-28 hours. Following the incubation, 5 ml of Kovac's reagent was added to the bacterial tryptophan broth. Red colored ring formation on the broth surface indicated that the organism was indole positive and absence of red colored ring showed that the bacterial isolate was negative.

2.8.4 Starch hydrolysis test (Seeley and Van Demark, 1981) [12]
The pure culture isolates were streaked on the nutrient agar Petri plates containing 2% starch and were kept for incubation at room temperature for the period of 2 days. After incubation, the Petri plates were flooded with Gram's iodine solution and observed for the hydrolysis of starch. The organism was considered as a positive indicator of starch hydrolysis when the Petri plates showed the clear zones surrounding the streaked colonies, whereas the absence of clear zones indicated that the organism was a negative indicator of starch hydrolysis.

2.8.5 Gelatin liquefaction test (McDade and Weaver, 1959) [10]
The pure cultures were stabbed separately into the test tube containing nutrient gelatin. The inoculated test tubes were kept for incubation at a temperature of 25ºC for 3-7 days and were checked every day for gelatin liquefaction. Subsequently, the test tubes were placed in the refrigerator or an ice bath for 10-20 minutes after which the changes were observed. The solid form of nutrient gelatin in the inoculated test tube indicated that the organism was negative in gelatin liquefaction reaction, whereas the presence of the liquid medium in the test tube showed that the organism had liquefied the gelatin and was a positive indicator of gelatin liquefaction test.

2.8.6 Carbon source utilization
The carbon source (sucrose) used in the medium (Jensen's medium) used for the growth of *Azotobacter* spp. was replaced with other carbon sources like rhamnose, starch, mannitol etc. The different isolates were grown in these alternative sources and the variation in their growth and development of colonies were observed.

2.8.7 Extracellular polysaccharide production
The slimy growth of the colonies indicated the production of extracellular polysaccharides by the bacterial isolates.

2.9 Determination of nitrogen fixing capacity (Jackson, 1967) [6]
The nitrogen fixing capacity of the isolates of *Azotobacter* was determined by micro kjeldahl method. For the purpose, the isolates were inoculated in Jensen's broth and kept for incubation for 10 days at the temperature of 37ºC. After the incubation period of 10 days, 10 ml of broth was taken and digested with the addition of 10 ml of concentrated sulphuric acid and 10 ml of 30% hydrogen peroxide. The appearance of clear liquid indicated that the sample was completely digested and was taken for the distillation process.

After completion of the digestion process, the volume of the digested mixture was made upto 100 ml with distilled water and allowed to cool for some time. Then these samples were taken in micro kjeldahl distillation apparatus. For the quick estimation of nitrogen fixing capacity by the bacterial isolates, 10 ml of the sample and 10 ml of 40% sodium hydroxide was added into the distillation flask. On the other hand, a conical flask containing 20-25 ml of 2% boric acid reagent with the addition of 3-4 drops of mixed indicator (prepared by dissolving 0.5 g bromo cresol green and 0.1 g methyl red in 100ml of 95% ethanol) was taken and connected to the condenser. It is important to note whether the tip of the condenser outlet is dipped into the flask containing boric acid. The entire distillation system was regulated by the continuous water flow for the purpose of heat control. Distillation was carried out for 9 minutes and the distilled off ammonia was collected in the conical flask containing solutions of boric acid and mixed indicator which was titrated against 0.02 N standard sulphuric acid until the disappearance of blue color from the solution.

The percentage of N_2 fixation of the isolates of pure culture was estimated by using the formula:

$$\% \text{ of } N_2 \text{ in sample} = \frac{\text{Sample titer} - \text{Blank titer}}{\text{Volume of sample in ml}} \text{ x Normality of } H_2SO_4 \text{ x } 14 \text{ x} 100$$

3. Results and Discussion
3.1 Colony characterization
The bacterial isolates exhibited wide variation in their colony characteristics under study (Fig. 1). Shapes of some of the bacterial colonies were circular, some were irregular in shape, some were oval and, some were round. In regard to size of colony, some colonies were small, some were moderate and, some were pinpoint. Margins of the colonies were mostly entire except one isolate. Elevation of the colonies in some of the isolates was raised, whereas in others it was flat. Pertaining to colony forms, colonies of some isolates exhibited circular form, while others exhibited rhizoid, irregular and regular forms. All the isolates were milky white in colour. In regard to consistency, colonies of all the isolates were translucent. (Table 1).

Table 1: Colony characteristics of *Azotobacter* isolates

Sr. No.	Isolate	Shape	Size	Margin	Elevation	Form	Color	Consistency
1.	PN 1	Circular	Small	Entire	Raised	Circular	Milky white	Translucent
2.	PN 2	Circular	Moderate	Entire	Raised	Circular	Milky white	Translucent
3.	PN 4	Circular	Small	Entire	Raised	Circular	Milky white	Translucent
4.	PN 6	Irregular	Small	Entire	Raised	Rhizoid	Milky white	Translucent
5.	PN 7	Circular	Pinpoint	Entire	Flat	Circular	Milky white	Translucent
6.	PN 8	Circular	Small	Entire	Flat	Rhizoid	Milky white	Translucent
7.	PN 9	Circular	Small	Entire	Flat	Circular	Milky white	Translucent

8.	PN 10	Circular	Small	Entire	Raised	Circular	Milky white	Translucent
9.	PN 11	Circular	Small	Entire	Raised	Circular	Milky white	Translucent
10.	PN 12	Circular	Moderate	Entire	Raised	Circular	Milky white	Translucent
11.	PN 14	Circular	Small	Entire	Raised	Circular	Milky white	Translucent
12.	PN 16	Circular	Pinpoint	Entire	Flat	Circular	Milky white	Translucent
13.	SL 1	Oval	Pinpoint	Entire	Flat	Circular	Milky white	Translucent
14.	SL 2	Circular	Moderate	Entire	Raised	Circular	Milky white	Translucent
15.	SL 5	Round	Pinpoint	Entire	Flat	Regular	Milky white	Translucent
16.	SL 6	Oval	Small	Entire	Raised	Circular	Milky white	Translucent
17.	SL 7	Circular	Pinpoint	Entire	Raised	Circular	Milky white	Translucent
18.	SL 8	Circular	Small	Entire	Raised	Circular	Milky white	Translucent
19.	SL 9	Circular	Moderate	Entire	Raised	Circular	Milky white	Translucent
20.	SL 10	Circular	Small	Entire	Raised	Circular	Milky white	Translucent
21.	SL 11	Circular	Small	Entire	Raised	Circular	Milky white	Translucent
22.	SL 13	Circular	Moderate	Entire	Flat	Circular	Milky white	Translucent
23.	SL 15	Oval	Moderate	Entire	Raised	Irregular	Milky white	Translucent
24.	ST 2	Circular	Small	Entire	Flat	Rhizoid	Milky white	Translucent
25.	ST 4	Circular	Small	Entire	Raised	Circular	Milky white	Translucent
26.	ST 5	Circular	Small	Entire	Raised	Circular	Milky white	Translucent
27.	ST 6	Round	Moderate	Entire	Flat	Rhizoid	Milky white	Translucent
28.	ST 8	Circular	Small	Entire	Raised	Circular	Milky white	Translucent
29.	ST 9	Circular	Small	Entire	Raised	Circular	Milky white	Translucent
30.	ST 11	Oval	Pinpoint	Entire	Flat	Circular	Milky white	Translucent
31.	ST 12	Round	Small	Entire	Raised	Circular	Milky white	Translucent
32.	AH 1	Circular	Small	Entire	Raised	Circular	Milky white	Translucent
33.	AH 3	Circular	Small	Entire	Raised	Circular	Milky white	Translucent
34.	AH 5	Circular	Small	Entire	Raised	Circular	Milky white	Translucent
35.	AH 6	Circular	Moderate	Entire	Raised	Circular	Milky white	Translucent
36.	AH 7	Circular	Small	Entire	Raised	Circular	Milky white	Translucent
37.	AH 9	Circular	Moderate	Entire	Flat	Rhizoid	Milky white	Translucent
38.	AH 11	Circular	Moderate	Entire	Flat	Irregular	Milky white	Translucent
39.	AH 13	Circular	Small	Entire	Raised	Circular	Milky white	Translucent
40.	AH 14	Oval	Large	Entire	Flat	Irregular	Milky white	Translucent
41.	AH 16	Round	Pinpoint	Entire	Flat	Circular	Milky white	Translucent
42.	AH 17	Circular	Small	Curled	Flat	Rhizoid	Milky white	Translucent
43.	AH 18	Oval	Small	Entire	Raised	Irregular	Milky white	Translucent
44.	AH 20	Oval	Moderate	Entire	Raised	Rhizoid	Milky white	Translucent
45.	AH 22	Circular	Small	Entire	Flat	Circular	Milky white	Translucent
46.	SG 1	Round	Small	Entire	Flat	Circular	Milky white	Translucent
47.	SG 2	Circular	Small	Entire	Flat	Circular	Milky white	Translucent
48.	SG 3	Round	Small	Entire	Flat	Circular	Milky white	Translucent
49.	SG 5	Oval	Moderate	Entire	Flat	Circular	Milky white	Translucent
50.	SG 7	Circular	Pinpoint	Entire	Flat	Circular	Milky white	Translucent

3.2 Cultural characterization

All the bacterial isolates were Gram negative in reaction (Fig. 2). Furthermore, the isolates under study varied in the production of pigments. Results regarding pigmentation clearly indicated that, the isolates produced the pigments from dull white to creamy white to dark brown color. (Fig. 3). In regard to production of extracellular polysaccharides, it ranged from low (+) to high (+++). (Fig. 4). Pertaining to arrangement of bacterial cells, it was noticed that some bacterial cells were arranged singly, some in pairs, some in chains and some were found in clumps. Regarding shape of the bacterial cell, all the bacteria were rod shaped. Furthermore, motility study clearly indicated that, some bacterial isolates were motile while others were non-motile. (Table 2).

Table 2: Cultural characteristics of *Azotobacter* isolates

Sr. No.	Isolate	Gram reaction	Cell arrangement	Pigmentation	Extracellular polysaccharide production	Motility test
1.	PN 1	-ve	Rod shaped in clusters	Dull white	+++	-ve
2.	PN 2	-ve	Rod shaped in clumps	White	++	-ve
3.	PN 4	-ve	Rod shaped in singles	White to light Yellow	+	+ve
4.	PN 6	-ve	Rod shaped in chains	Creamy white	+++	+ve
5.	PN 7	-ve	Rod shaped in singles	Dark brown	+	+ve
6.	PN 8	-ve	Rod shaped in pairs	Creamy white	+++	+ve
7.	PN 9	-ve	Rod shaped in pairs	Dull white	+	-ve
8.	PN 10	-ve	Rod shaped in pairs	White to light Yellow	+	+ve
9.	PN 11	-ve	Rod shaped in singles	Dull white	+	+ve
10.	PN 12	-ve	Rod shaped in singles	Creamy white	++	+ve
11.	PN 14	-ve	Rod shaped in singles	Creamy white	++	-ve

12.	PN 16	-ve	Rod shaped in pairs	Creamy white	++	+ve
13.	SL 1	-ve	Rod shaped in singles	Creamy white	++	+ve
14.	SL 2	-ve	Rod shaped in singles	Creamy white	+++	+ve
15.	SL 5	-ve	Rod shaped in chains	Creamy white	+++	+ve
16.	SL 6	-ve	Rod shaped in singles	Creamy white	++	-ve
17.	SL 7	-ve	Rod shaped in pairs	Creamy white	+++	+ve
18.	SL 8	-ve	Rod shaped in singles	Creamy white	+++	+ve
19.	SL 9	-ve	Rod shaped in singles	Creamy white	+++	+ve
20.	SL 10	-ve	Rod shaped in clusters	Dull white	++	-ve
21.	SL 11	-ve	Rod shaped in singles	Creamy white	++	-ve
22.	SL 13	-ve	Rod shaped in singles	White to light Yellow	+	+ve
23.	SL 15	-ve	Rod shaped in singles	Creamy white	++	-ve
24.	ST 2	-ve	Rod shaped in clusters	White to light Yellow	+	+ve
25.	ST 4	-ve	Rod shaped in singles	Creamy white	++	+ve
26.	ST 5	-ve	Rod shaped in clusters	Creamy white	++	+ve
27.	ST 6	-ve	Rod shaped in singles	Dull white	+	+ve
28.	ST 8	-ve	Rod shaped in singles	Creamy white	++	-ve
29.	ST 9	-ve	Rod shaped in singles	Creamy white	++	-ve
30.	ST 11	-ve	Rod shaped in pairs	Creamy white	++	+ve
31.	ST 12	-ve	Rod shaped in singles	Creamy white	+++	+ve
32.	AH 1	-ve	Rod shaped in pairs	Dull white	++	+ve
33.	AH 3	-ve	Rod shaped in singles	Dull white	++	+ve
34.	AH 5	-ve	Rod shaped in singles	Dull white	+	+ve
35.	AH 6	-ve	Rod shaped in chains	Creamy white	+++	+ve
36.	AH 7	-ve	Rod shaped in clusters	Creamy white	++	+ve
37.	AH 9	-ve	Rod shaped in singles	Creamy white	+++	+ve
38.	AH 11	-ve	Rod shaped in clumps	Creamy white	++	+ve
39.	AH 13	-ve	Rod shaped in singles	Dull white	+	+ve
40.	AH 14	-ve	Rod shaped in clusters	Creamy white	++	+ve
41.	AH 16	-ve	Rod shaped in pairs	Creamy white	+++	+ve
42.	AH 17	-ve	Rod shaped in singles	Creamy white	+++	+ve
43.	AH 18	-ve	Rod shaped in singles	Creamy white	++	+ve
44.	AH 20	-ve	Rod shaped in singles	Creamy white	++	-ve
45.	AH 22	-ve	Rod shaped in singles	Creamy white	+++	+ve
46.	SG 1	-ve	Rod shaped in pairs	Creamy white	+	+ve
47.	SG 2	-ve	Rod shaped in clusters	Creamy white	++	+ve
48.	SG 3	-ve	Rod shaped in clumps	White to light Yellow	++	+ve
49.	SG 5	-ve	Rod shaped in singles	White to light Yellow	+	+ve
50.	SG 7	-ve	Rod shaped in singles	Dull white	++	-ve

3.3 Biochemical characterization

The results of the investigation depicted in Table 3 clearly indicated that, all the isolates were catalase positive, oxidase negative and showed positive reaction for gelatin liquefaction (Fig. 5) and indole formation. In regard to starch hydrolysis, some isolates indicated positive reaction, whereas some were negative for starch hydrolysis test. (Fig. 6).

Table 3: Biochemical characteristics of *Azotobacter* isolates (tests)

Sr. No.	Isolate	Catalase	Oxidase	Indole formation	Starch hydrolysis	Gelatin liquefaction
1.	PN 1	+ve	-ve	+ve	+ve	+ve
2.	PN 2	+ve	-ve	+ve	+ve	+ve
3.	PN 4	+ve	-ve	+ve	-ve	+ve
4.	PN 6	+ve	-ve	+ve	+ve	+ve
5.	PN 7	+ve	-ve	+ve	-ve	+ve
6.	PN 8	+ve	-ve	+ve	-ve	+ve
7.	PN 9	+ve	-ve	+ve	+ve	+ve
8.	PN 10	+ve	-ve	+ve	+ve	+ve
9.	PN 11	+ve	-ve	+ve	+ve	+ve
10.	PN 12	+ve	-ve	+ve	-ve	+ve
11.	PN 14	+ve	-ve	+ve	+ve	+ve
12.	PN 16	+ve	-ve	+ve	-ve	+ve
13.	SL 1	+ve	-ve	+ve	+ve	+ve
14.	SL 2	+ve	-ve	+ve	-ve	+ve
15.	SL 5	+ve	-ve	+ve	+ve	+ve
16.	SL 6	+ve	-ve	+ve	+ve	+ve
17.	SL 7	+ve	-ve	+ve	-ve	+ve
18.	SL 8	+ve	-ve	+ve	-ve	+ve
19.	SL 9	+ve	-ve	+ve	+ve	+ve

20.	SL 10	+ve	-ve	+ve	-ve	+ve
21.	SL 11	+ve	-ve	+ve	+ve	+ve
22.	SL 13	+ve	-ve	+ve	+ve	+ve
23.	SL 15	+ve	-ve	+ve	-ve	+ve
24.	ST 2	+ve	-ve	+ve	-ve	+ve
25.	ST 4	+ve	-ve	+ve	-ve	+ve
26.	ST 5	+ve	-ve	+ve	-ve	+ve
27.	ST 6	+ve	-ve	+ve	-ve	+ve
28.	ST 8	+ve	-ve	+ve	+ve	+ve
29.	ST 9	+ve	-ve	+ve	-ve	+ve
30.	ST 11	+ve	-ve	+ve	-ve	+ve
31.	ST 12	+ve	-ve	+ve	-ve	+ve
32.	AH 1	+ve	-ve	+ve	-ve	+ve
33.	AH 3	+ve	-ve	+ve	-ve	+ve
34.	AH 5	+ve	-ve	+ve	-ve	+ve
35.	AH 6	+ve	-ve	+ve	+ve	+ve
36.	AH 7	+ve	-ve	+ve	-ve	+ve
37.	AH 9	+ve	-ve	+ve	-ve	+ve
38.	AH 11	+ve	-ve	+ve	-ve	+ve
39.	AH 13	+ve	-ve	+ve	+ve	+ve
40.	AH 14	+ve	-ve	+ve	-ve	+ve
41.	AH 16	+ve	-ve	+ve	-ve	+ve
42.	AH 17	+ve	-ve	+ve	-ve	+ve
43.	AH 18	+ve	-ve	+ve	+ve	+ve
44.	AH 20	+ve	-ve	+ve	-ve	+ve
45.	AH 22	+ve	-ve	+ve	-ve	+ve
46.	SG 1	+ve	-ve	+ve	+ve	+ve
47.	SG 2	+ve	-ve	+ve	+ve	+ve
48.	SG 3	+ve	-ve	+ve	+ve	+ve
49.	SG 5	+ve	-ve	+ve	-ve	+ve
50.	SG 7	+ve	-ve	+ve	-ve	+ve

3.4 Carbon source utilization

The bacterial isolates obtained from different soil samples were appraised for their ability to utilize different carbon sources in the medium like sucrose, starch, mannitol and rhamnose. Results of the present study presented in Table 4, explicitly indicated that, all the isolates showed a good bacterial growth in the Petri plate, where sucrose was used as a carbon source and no growth was observed where starch, mannitol and rhamnose were used as a source of carbon.

Table 4: Carbon source utilization by *Azotobacter*

Sr. No.	Isolate	Sucrose	Starch	Mannitol	Rhamnose
1.	PN 1	+ve	-ve	-ve	-ve
2.	PN 2	+ve	-ve	-ve	-ve
3.	PN 4	+ve	-ve	-ve	-ve
4.	PN 6	+ve	-ve	-ve	-ve
5.	PN 7	+ve	-ve	-ve	-ve
6.	PN 8	+ve	-ve	-ve	-ve
7.	PN 9	+ve	-ve	-ve	-ve
8.	PN 10	+ve	-ve	-ve	-ve
9.	PN 11	+ve	-ve	-ve	-ve
10.	PN 12	+ve	-ve	-ve	-ve
11.	PN 14	+ve	-ve	-ve	-ve
12.	PN 16	+ve	-ve	-ve	-ve
13.	SL 1	+ve	-ve	-ve	-ve
14.	SL 2	+ve	-ve	-ve	-ve
15.	SL 5	+ve	-ve	-ve	-ve
16.	SL 6	+ve	-ve	-ve	-ve
17.	SL 7	+ve	-ve	-ve	-ve
18.	SL 8	+ve	-ve	-ve	-ve
19.	SL 9	+ve	-ve	-ve	-ve
20.	SL 10	+ve	-ve	-ve	-ve
21.	SL 11	+ve	-ve	-ve	-ve
22.	SL 13	+ve	-ve	-ve	-ve
23.	SL 15	+ve	-ve	-ve	-ve
24.	ST 2	+ve	-ve	-ve	-ve
25.	ST 4	+ve	-ve	-ve	-ve
26.	ST 5	+ve	-ve	-ve	-ve

27.	ST 6	+ve	-ve	-ve	-ve
28.	ST 8	+ve	-ve	-ve	-ve
29.	ST 9	+ve	-ve	-ve	-ve
30.	ST 11	+ve	-ve	-ve	-ve
31.	ST 12	+ve	-ve	-ve	-ve
32.	AH 1	+ve	-ve	-ve	-ve
33.	AH 3	+ve	-ve	-ve	-ve
34.	AH 5	+ve	-ve	-ve	-ve
35.	AH 6	+ve	-ve	-ve	-ve
36.	AH 7	+ve	-ve	-ve	-ve
37.	AH 9	+ve	-ve	-ve	-ve
38.	AH 11	+ve	-ve	-ve	-ve
39.	AH 13	+ve	-ve	-ve	-ve
40.	AH 14	+ve	-ve	-ve	-ve
41.	AH 16	+ve	-ve	-ve	-ve
42.	AH 17	+ve	-ve	-ve	-ve
43.	AH 18	+ve	-ve	-ve	-ve
44.	AH 20	+ve	-ve	-ve	-ve
45.	AH 22	+ve	-ve	-ve	-ve
46.	SG 1	+ve	-ve	-ve	-ve
47.	SG 2	+ve	-ve	-ve	-ve
48.	SG 3	+ve	-ve	-ve	-ve
49.	SG 5	+ve	-ve	-ve	-ve
50.	SG 7	+ve	-ve	-ve	-ve

3.5 Estimation of nitrogen fixing capacity

The dinitrogen fixing ability of the isolates under study was appraised using micro Kjeldahl method. Results pertaining to dinitrogen fixing ability of the bacterial isolates, depicted in Table 5, substantiated that all the 50 bacterial isolates had the ability to fix atmospheric nitrogen. The nitrogen fixation capacity of the bacterial isolates varied widely and ranged between 2.8 and 34.0 mg N /g of sucrose consumed. Among all the isolates, 23 isolates fixed nitrogen more than 10 mg / g sucrose. The results further unveiled that 8 bacterial isolates had nitrogen fixing capacity greater than 20 mg N / g of sucrose and 4 bacterial isolates had capacity to fix nitrogen greater than 30 mg N / g of sucrose. Amongst them, isolate PN7 fixed 34.0 mg N / g of sucrose, PN 8 fixed 31.0 mg N / g of sucrose, PN 2 30.8 mg N / g of sucrose and AH 1 fixed 30.8 mg N / g of sucrose. On the other hand, the isolates ST 6, SG 5, AH 18, AH 6 (2.6 mg N / g of sucrose) followed by the isolates AH 17, ST 9, PN 14 (5.6 mg N / g of sucrose) and the isolate AH 22, SG 2 (6.0 mg N / g of sucrose) were found to fix nitrogen less than 10 mg / g of sucrose. Thus, 15 bacterial isolates were found to fix less than 10 mg N / g of sucrose. Consequently, wide variation relating to nitrogen fixing ability was observed in the isolates obtained in the study.

Table 5: Nitrogen fixing efficiency of *Azotobacter* isolates

Sr. No.	Isolate	Amount of N_2 fixed (mg/g of sucrose)	Sr. No.	Isolate	Amount of N_2 fixed (mg/g of sucrose)
1.	PN 1	8.4	26.	ST 5	14.0
2.	PN 2	30.8	27.	ST 6	2.8
3.	PN 4	17.0	28.	ST 8	20.0
4.	PN 6	28.0	29.	ST 9	5.6
5.	PN 7	34.0	30.	ST 11	11.0
6.	PN 8	31.0	31.	ST 12	11.2
7.	PN 9	18.2	32.	AH 1	30.8
8.	PN 10	8.4	33.	AH 3	28.0
9.	PN 11	14.0	34.	AH 5	11.2
10.	PN 12	16.8	35.	AH 6	2.8
11.	PN 14	5.6	36.	AH 7	8.0
12.	PN 16	19.6	37.	AH 9	8.4
13.	SL 1	17.0	38.	AH 11	11.2
14.	SL 2	20.0	39.	AH 13	14.0
15.	SL 5	14.0	40.	AH 14	11.0
16.	SL 6	25.2	41.	AH 16	11.0
17.	SL 7	11.2	42.	AH 17	5.6
18.	SL 8	11.2	43.	AH 18	2.8
19.	SL 9	8.4	44.	AH 20	8.4
20.	SL 10	28.0	45.	AH 22	6.0
21.	SL 11	17.0	46.	SG 1	22.4
22.	SL 13	22.0	47.	SG 2	6.0
23.	SL 15	22.4	48.	SG 3	14.0
24.	ST 2	14.0	49.	SG 5	2.8
25.	ST 4	11.2	50.	SG 7	22.4

3.6 Identification of isolates based on various tests

On the basis of results of biochemical tests and morphological observations, the isolates were identified as given below in the Table 6.

Table 6: Identification of isolates

Sr. No.	Isolate	Motility test	Starch	Mannitol	Rhamnose	Organism identified
1.	PN 1	-ve	-ve	-ve	-ve	*A. beijerinckii*
2.	PN 2	-ve	-ve	-ve	-ve	*A. beijerinckii*
3.	PN 4	+ve	-ve	-ve	-ve	*A. paspali*
4.	PN 6	+ve	-ve	-ve	-ve	*A. paspali*
5.	PN 7	+ve	-ve	-ve	-ve	*A. paspali*
6.	PN 8	+ve	-ve	-ve	-ve	*A. paspali*
7.	PN 9	-ve	-ve	-ve	-ve	*A. beijerinckii*
8.	PN 10	+ve	-ve	-ve	-ve	*A. paspali*
9.	PN 11	+ve	-ve	-ve	-ve	*A. paspali*
10.	PN 12	+ve	-ve	-ve	-ve	*A. paspali*
11.	PN 14	-ve	-ve	-ve	-ve	*A. beijerinckii*
12.	PN 16	+ve	-ve	-ve	-ve	*A. paspali*
13.	SL 1	+ve	-ve	-ve	-ve	*A. paspali*
14.	SL 2	+ve	-ve	-ve	-ve	*A. paspali*
15.	SL 5	+ve	-ve	-ve	-ve	*A. paspali*
16.	SL 6	-ve	-ve	-ve	-ve	*A. paspali*
17.	SL 7	+ve	-ve	-ve	-ve	*A. paspali*
18.	SL 8	+ve	-ve	-ve	-ve	*A. paspali*
19.	SL 9	+ve	-ve	-ve	-ve	*A. paspali*
20.	SL 10	-ve	-ve	-ve	-ve	*A. beijerinckii*
21.	SL 11	-ve	-ve	-ve	-ve	*A. beijerinckii*
22.	SL 13	+ve	-ve	-ve	-ve	*A. paspali*
23.	SL 15	-ve	-ve	-ve	-ve	*A. beijerinckii*
24.	ST 2	+ve	-ve	-ve	-ve	*A. paspali*
25.	ST 4	+ve	-ve	-ve	-ve	*A. paspali*
26.	ST 5	+ve	-ve	-ve	-ve	*A. paspali*
27.	ST 6	+ve	-ve	-ve	-ve	*A. paspali*
28.	ST 8	-ve	-ve	-ve	-ve	*A. beijerinckii*
29.	ST 9	-ve	-ve	-ve	-ve	*A. beijerinckii*
30.	ST 11	+ve	-ve	-ve	-ve	*A. paspali*
31.	ST 12	+ve	-ve	-ve	-ve	*A. paspali*
32.	AH 1	+ve	-ve	-ve	-ve	*A. paspali*
33.	AH 3	+ve	-ve	-ve	-ve	*A. paspali*
34.	AH 5	+ve	-ve	-ve	-ve	*A. paspali*
35.	AH 6	+ve	-ve	-ve	-ve	*A. paspali*
36.	AH 7	+ve	-ve	-ve	-ve	*A. paspali*
37.	AH 9	+ve	-ve	-ve	-ve	*A. paspali*
38.	AH 11	+ve	-ve	-ve	-ve	*A. paspali*
39.	AH 13	+ve	-ve	-ve	-ve	*A. paspali*
40.	AH 14	+ve	-ve	-ve	-ve	*A. paspali*
41.	AH 16	+ve	-ve	-ve	-ve	*A. paspali*
42.	AH 17	+ve	-ve	-ve	-ve	*A. paspali*
43.	AH 18	+ve	-ve	-ve	-ve	*A. paspali*
44.	AH 20	-ve	-ve	-ve	-ve	*A. beijerinckii*
45.	AH 22	+ve	-ve	-ve	-ve	*A. paspali*
46.	SG 1	+ve	-ve	-ve	-ve	*A. paspali*
47.	SG 2	+ve	-ve	-ve	-ve	*A. paspali*
48.	SG 3	+ve	-ve	-ve	-ve	*A. paspali*
49.	SG 5	+ve	-ve	-ve	-ve	*A. paspali*
50.	SG 7	-ve	-ve	-ve	-ve	*A. beijerinckii*

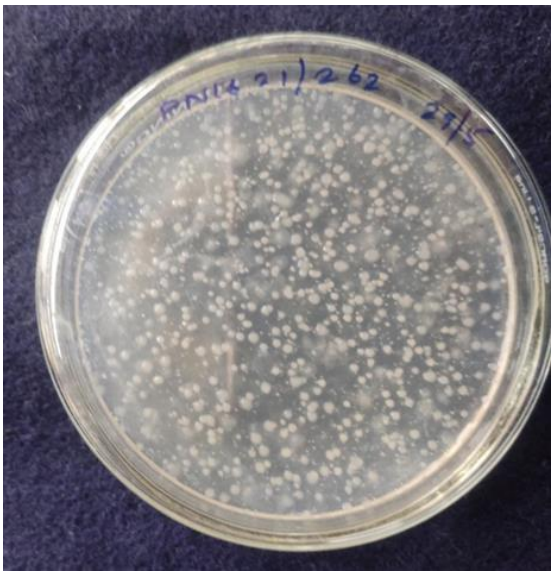

Fig 1: Colony characterization

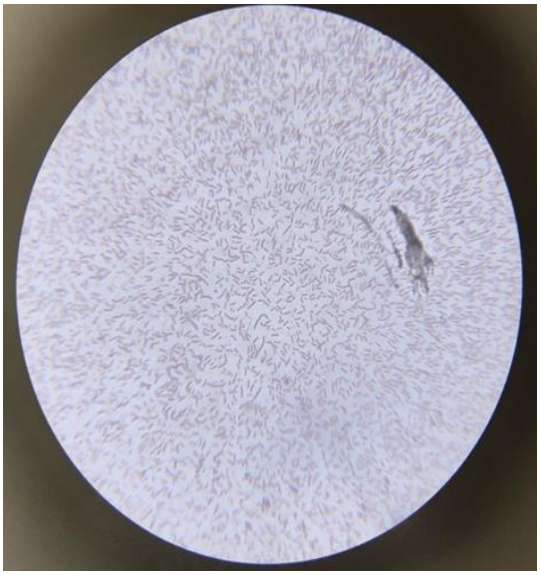

Fig 2: Gram staining

Fig 3: Pigmentation

Fig 4: Extracellular polysaccharide production

Fig 5: Gelatin liquefaction

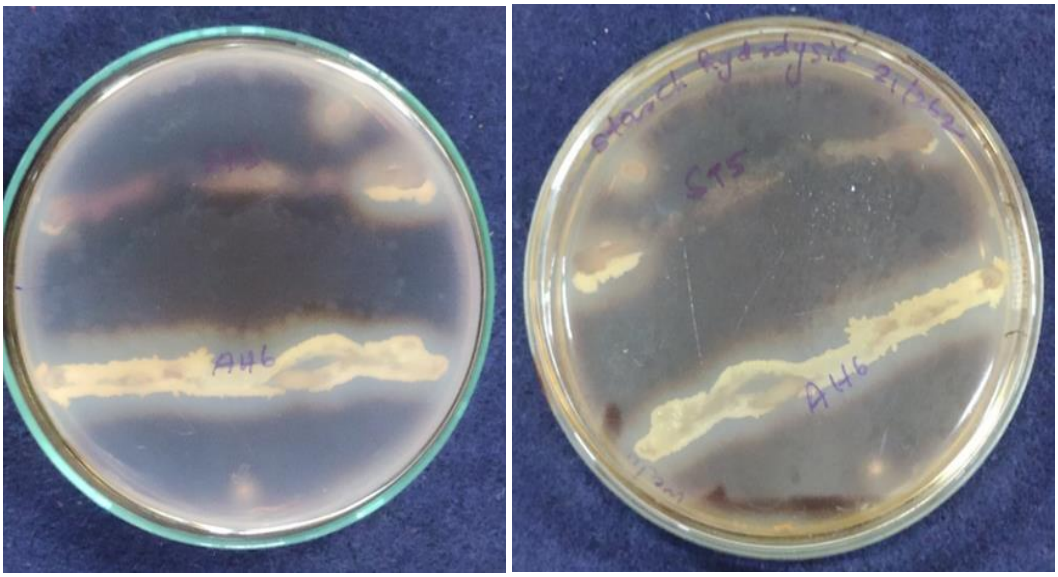

Fig 6: Starch hydrolysis

4. Conclusion

From the results of morphological, cultural and biochemical tests, it was evident that all the bacterial isolates obtained were *Azotobacter*, since all the isolates were negative in Gram staining reaction and positive in catalase, indole formation and gelatin liquefaction tests. The colonies of the isolates were small to large, cells were rod shaped arranged in singles or pairs with marked pleomorphism in size, shape, margin, elevation and form producing dull white to dark brown pigments and extracellular polysaccharides, confirmed that the isolates belonged to *Azotobacter* species.

To get a better clarification for the confirmation of the bacterial species, biochemical characterization of the isolates were taken into account. All the bacterial isolates showed positive indicator for catalase test, indole production and gelatin liquefaction tests, while some isolates were positive and negative for starch hydrolysis test and negative for oxidase test. Thus, it was clear and evident that these characters resembled the properties of *Azotobacter* species.

Motility test and carbon source utilization test (Upadhyay *et al.* 2015) [15] helped in further identification of the bacterial isolates. As per the Bergey's manual of Systematic Bacteriology (2001-2012), the non- motile *Azotobacter* isolates belongs to *Azotobacter beijerinckii* whereas motile *Azotobacter* isolates belongs to *A.chrococcum, A. vinelandii* and *A. paspali* species. Also, *Azotobacter* isolate not growing in the carbon sources- starch, mannitol and rhamnose belongs to *A. beijerinckii* and *A. paspali.* In adherence to these facts, the isolates of *Azotobacter beijerinckii* and *Azotobacter paspali* were identified.

Thus, among the 50 isolates obtained, 11 isolates belonged to *A. beijerinckii* and remaining 39 isolates belonged to *A. paspali* showing several variation in morphological, biochemical characteristics and nitrogen fixing capacity. Both the species *Azotobacter beijerinckii* and *Azotobacter paspali* were found to be fixing atmospheric nitrogen in similar ranges. The isolates PN 7, PN 8, AH 1 (*A. paspali)* and PN 2 (*A. beijerinckii)* were found to be fixing nitrogen greater than 30 mg N / g of sucrose whereas the isolates ST 6, AH 6, AH 18, AH 7, AH 9, AH 17 (*A. paspali)* and AH 20 (*A. beijerinckii)* were found to be fixing atmospheric nitrogen lesser than 10 mg N / g of sucrose consumed.

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