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RESEARCH ARTICLE

Characterization of *Azotobacter beijerinckii* for PGP properties isolated from tomato rhizosphere of Darjeeling Hills

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Abstract

The rhizospheric soil is the habitat of a various kind of microorganisms that play a key role in the enhancement of plant growth. The present study was carried out to screen the abundance of free-living dinitrogen-fixing microorganisms from the tomato rhizosphere of Darjeeling Hills. The bacteria that could fix nitrogen from the atmosphere and grow on media devoid of nitrogen were isolated. One of the potent strains was selected and identified as *Azotobacter beijerinckii* through molecular characterization using 16S rRNA homology at NCMR-NCCS, Pune. *In-vitro* production of IAA was 27 (µg/mL), Gibberellic acid was 48 (µg/mL) and the Phosphate solubilization index (SI) was 2.86. The plant growth promotion test was performed on rice seeds using the strain. The results showed that the % increase in germination over control was 7.52%.

Keywords

Azotobacter ; Indole Acetic Acid; inorganic phosphate; Darjeeling Hills; biofertilizer

Introduction

Plant growth promoting rhizobacteria are free-living soil microorganisms that naturally colonize the rhizospheric zone of plant roots. Plant growth promoting rhizobacteria (PGPR) can directly or indirectly enhance the quality of plant growth. The rhizospheric interactions between plants and microbes determine the health of plants and the fertility of the soil (1).

Over the past few decades, a wide range of bacteria, comprising the species *Azotobacter, Azospirillum, Pseudomonas, Burkholderia, Bacillus, Enterobacter, Klebsiella, Alcaligens, Arthobacter* and *Serratia*, have been discovered and claimed to promote plant development (2-4). The creation of novel compounds and characteristics that aid in plant growth has made microorganisms an important subject of study. Different types of microbes, including *Azotobacter* (5) and *Pseudomonas* (6), create these compounds. Numerous bacterial strains generate siderophores or organic acids, which inhibit the growth of harmful pathogens and raise the availability of iron (7, 8). The synthesis of phytohormones, which aid in nitrogen fixation, phosphorus release, ammonia production and siderophore production are few examples of promoting plant development (9).

Azotobacter spp. are aerobic, gram-negative, free-living, oval or spherical-shaped and soil-dwelling bacteria (10, 11). During their resting phase, they form a thick-walled cyst that shields them from the harsh

environment (12). A Dutch scientist and botanist named Beijerinck first discovered the *Azotobacter* in 1917 (13). There are 7 species in the genus *Azotobacter: Azotobacter chroococcum, A. beijerinckii, A. armeniacus, A. vinelandii A. nigricans, A. salinestris* and *A. paspali* (14, 15). They vary in size from 2 to 10 µm in length and 1 to 2 µm in width (16).

In addition to fixing atmospheric N_2 under free-living conditions, studies reported that *Azotobacter* shown to have a variety of other plant growth-promoting properties, including phosphate solubilization and the synthesis of plant growth regulators (PGRs) like auxins, gibberellins, cytokinins, vitamins and amino acids. It has been found that several Plant growth-promoting rhizobacteria produce auxins, which are essential for controlling root development and architecture (17). These also produce siderophores and antibiotics that impede the growth of phytopathogens (18-20). Furthermore, the application of *Azotobacter* has shown promise as a putative agent for converting unused land to fertile land and has become useful in soil reclamation (21). The earlier studies reported that the association of *Azotobacter* species with host plant Canola (*Brassica napus*) secreted growth substances like IAA, Gibberellin, Kinetin, phosphate and antioxidant activity which helped in enhancing plant growth and survival (22, 23).

Species of *Azotobacter* can be found in a variety of habitats, including soil, water, the surfaces of leaves, roots and other plant surfaces. Some species can also be found in the polar and tropical zones. In varied soils, their frequency varies. They are more common in neutral to alkaline soils than in acidic soils, where they are infrequent (24).

The main purpose of our research study was to isolate *Azotobacter* species from tomato rhizosphere of Darjeeling hills having the ability to produce growth hormones, dinitrogen fixing and solubilize phosphate which will help in the development of potent indigenous biofertilizer to be used in upland agricultural system. A previous study reported that in the temperate region of Sikkim, a field experiment using 2 certified nitrogen-fixing biofertilizers (*Azotobacter chroococcum* and *Azospirillum brasilense*) with maize as the test crop did not improve plant performance (25). For relatively higher altitudes, the isolation and identification of these native microorganisms should take precedence. The present study on the characterization of PGP properties of *Azotobacter beijerinckii* specifically in the context of the Darjeeling Hills' unique environmental conditions could lead to the development of native biofertilizer. Understanding the PGP potential of these strains can lead to the development of sustainable agricultural practices that promote plant growth and reduce dependency on chemical fertilizers, contributing to food security and environmental protection.

Materials and Methods

Collection of soil sample

The soil samples required for the study were collected from a depth of up to 15 cm from the rhizospheric region of tomato fields from different sites in Darjeeling hills. Care should be taken to collect soil i.e., not too dry or wet. Optimal moisture content is required for the survival of *Azotobacter*. Multiple sampling points within the area were selected to ensure representatives. About 10 to 15 g of soil samples were collected with the help of a sterile spatula in sterile polybags and brought to the laboratory for further analysis. Before proceeding further for analysis, soil samples were sieved to remove all organic matter and unwanted materials. Soil samples from each site were mixed and composited before analysis.

Isolation of free-living nitrogen-fixing bacteria from tomato rhizosphere

Isolation was done on the Ashby's medium by serial dilution method. Under aseptic conditions, 10 g of soil samples were suspended in ninety (90 mL) of sterile double-distilled water and considered as 10^{-1} dilitions. Serial dilutions were made until the 10^{-5} dilutions were achieved. From 10^{-4} and 10^{-5} dilutions enrichment was done by inoculating 10 mL pre-sterilized Ashby's broth [20.0 g Mannitol, 0.2 g potassium phosphate dibasic, 0.2 g magnesium sulfate, 0.2 g sodium chloride, 0.1 g potassium sulfate, 5.0 g calcium carbonate, distilled water 1000 mL, pH adjusted to 7.4±0.2] with 0.1 mL of sample. Repeated transfer of 0.1 mL was made after a few days of growth to fresh pre-sterilized Ashby's broth. From 10^{-4} and 10^{-5} dilutions spread plates were made on Ashby's agar medium [Ashby broth with 15 g agar (for solidification)] and incubated at 28±° C for 7 days for the development of visible colonies. Bacterial colonies showing slimy, glistening mucoid colonies and producing pigments were selected, purified and sub-cultured in Ashby's slant for further studies. The morphological and biochemical features of the bacterial isolates were used for preliminary identification following Bergey's manual of determinative bacteriology (26). The potent isolate was sent to NCMR- NCCS Pune, for molecular characterization using the 16SrRNA method.

Morphological characterization

The morphological and growth parameters of selected isolate TDH-01, such as color, shape and size, appearance, colony diameter, margin and Gram nature were determined following standard protocol. The strain was inoculated on Ashby agar plates and incubated at 28 ± °C for 48 h. The incubation temperature for the evaluation of other *in vitro* properties of *Azotobacter* was also 28± °C. The highest density of *Azotobacter* spp occurs around 28 °C and the optimum temperature for the growth of *Azotobacter* spp was around 30 °C as reported in previous studies (27).

Biochemical characterization and Measurement of pH

The selected isolate was characterized based on biochemical features like carbohydrate fermentation, Indole test, citrate utilization, oxidase test, catalase, hydrogen sulfide production and Nitrate reduction as per following standard methods (28). Change in the pH of the inoculated broth was measured with the help of the pH meter after 7 days of incubation.

Characterization of the isolate for PGP traits

Screening of Nitrogen-fixing activity

The nitrogen-fixing activities of the bacterial isolate were observed by using a glucose nitrogen-free mineral (GNFM) agar medium containing BTB. The bacterial isolates were inoculated into glucose nitrogen-free mineral media (GNFM) prepared plates and incubated for seven days at 28 ºC and the change in the color of colonies was determined (29).

Indole acetic acid production

The bacterial isolate was cultured in Jensen Medium with 100 mg/L of tryptophan as supplement and was incubated for 7 days at 28 ºC. 2 mL of culture was taken out from each tube after 7 days of incubation period and it was centrifuged for 15 min at 10000 rpm. A new fresh tube was filled with 1 mL of supernatant fluid and 100 µL of 10 mM orthophosphoric acid and 2 mL of reagent consisting of 1 mL of 0.5 $FeCl_3$ in 50 mL of 35% $HClO_4$ were added. Absorbance of the developed pink color was read at 530 nm after 25 min. The IAA concentration in the culture was determined by using a calibration curve of pure IAA as a standard, following linear regression analysis (30).

Phosphate solubilizing activity

Solubilization of inorganic phosphate by the isolate was done on Pikovskaya's agar (PKV) by the Agar spot method. Pikovskaya's agar plates were inoculated with the bacterial isolate following the Agar Spot method and were incubated at 28 °C for 7 days. The bacterial isolate produces a transparent zone of clearing around the colonies indicating solubilization of phosphate by isolates (12). The diameter of the colony of phosphate solubilized by bacterial isolate (halo zones) was measured with the help of a metric scale. Solubilization efficiency and solubilization index were also calculated using the formula. Solubilization Efficiency (SE) = Solubilization diameter/ Colony diameter x 100 and Solubilization Index (SI) = Colony diameter+ Halo diameter/ Colony diameter (31-33).

Quantification of Phosphate solubilization

Soluble phosphate content in the cell-free extract was estimated *in-vitro* in a Pikovskaya's broth supplemented with a known quantity of tricalcium phosphate as a substrate. Control without bacterial inoculation was also maintained. The inoculated broth was incubated for 7 days at 28 °C and centrifuged for 10-15 min at 10000 rpm. Phosphorus in the supernatant was determined by using Barton's reagent (34).

Gibberellic Acid production

The bacterial isolate was grown in Ashby's nitrogen-free broth at 28 °C for 3 days and the amount of gibberellic acid was estimated by a spectrophotometric method using phosphomolybdic reagent. After incubation, the sample was centrifuged at 3000 rpm for 10 min. In a volumetric flask of 25 mL, 1 mL of supernatant and 15 mL of phosphomolybdic acid reagent were added. The content of the flask was thoroughly mixed and kept in a water bath (boiling) for 60 min. The flasks was taken out, their temperature was allowed to cool to room temperature and distilled water was added to bring the final volume to 25 mL. Using distilled water as a blank, the absorbance of the color developed was measured at 780 nm in a spectrophotometer. Using a standard curve made from the gibberellic acid standard solution, the concentration was ascertained (35).

Screening of Siderophore Production

Bacterial isolate was assayed for the production of siderophore qualitatively by Chrome azurol S (CAS) agar medium. The prepared CAS agar plate was spotted by bacterial isolate and incubated for 3 days at 28 °C. Siderophore production was indicated by the development of a halo zone around the colonies (36).

Hydrogen cyanide production

Production of hydrogen cyanide was tested by inoculating the bacterial isolate in ashby agar plate with 4.4 g/L glycine (supplemented). Whatman paper No. 2 immersed with picric acid (0.5%) and sodium carbonate (2%) was overlaid at the inoculated plate. The plates were incubated for 2-4 days at 28 °C. Production of hydrogen cyanide was indicated by the changes in the color of the filter paper saturated with the reagent from yellow to orange-brown (37).

Ammonia production

Peptone water was used for testing the production of ammonia by bacterial isolate. After inoculating the bacterial isolate in peptone water, it was incubated at 28 ° C for 48 h. and after that Nesseler reagent (0.5 mL) was added to the culture. Production of ammonia was indicated by the development of yellow to dark orange color (30).

Molecular Identification of bacterial strain TDH-01

The bacterial isolate TDH-01 was sent to the National Centre for Microbial Resource (NCMR), National Centre for Cell Science sequencing facility, located in Pune for molecular identification using 16SrRNA method. The isolation of genomic DNA was done following the standard phenol/ chloroform extraction method (38), followed by PCR amplification of the 16S rRNA gene using universal primers 16F27 [5'-CCA GAG TTT GAT CMT GGC TCA G-3'] and 16R1492 [5'-TAC GGY TAC CTT GTT ACG ACT T-3'].

In vitro Plant growth promotion test on Rice seeds using TDH-01 strain

Rice seeds were surface sterilized with $HgCl_2$(0.1%) for 3 mins. and was successively washed with sterile distilled water. One filter paper was placed in each Petri dish. The sterile distilled water was used to moisten the filter paper. The Petri dish with 15 seeds in each was kept for 24 h in the dark at 25 ºC and then treated with 2 mL of bacterial culture at a cell density of about 1x 10^4cfu/ mL. in each petri dish and incubated at room temperature. Control seeds were treated with a sterilized medium instead of bacterial culture. Germination was recorded each day up to 14 days. The germination percentage and seedling vigor index were calculated (39).

The percentage of germination and seedling vigor index was calculated as:

$$\text{Germination \% (GP)} = \frac{\text{Total no. of seeds germinated}}{\text{Total no. of seeds …}} \times 100 \quad \text{.....(i)}$$

Seedling vigor index (SVI) was calculated using the formula

Seedling vigor Index (SVI) = [(mean root length + mean shoot length) x GP].....(ii)

Greenhouse Pot experiment

Rice seeds were thoroughly washed with distilled water (sterile) and treated with Ashby broth having confluent growth of bacterial isolate for 2 h. Garden soil used for agricultural purposes was sterilized and filled in earthen pots. The treated seeds of rice were germinated in earthen pots containing sterilized soil and kept for 15 days under greenhouse conditions. Watering was done regularly. The measurements were recorded for the 5th, 10th and 15th days. Root length and shoot length were measured in scale.

Statistical analysis

The observations were recorded and tabulated. The final observations were analyzed statistically using Microsoft Excel.

Results and discussion

Morphological characterization

Bacterial strain isolated from the tomato field of Darjeeling Hills shows the cell shape blunt rod to ellipsoid in morphology. Cells were up to 2-4 µm in length. Colonies were found to be smooth, glistening, mucoid and black pigments produced in old culture (Fig. 1). The isolate was gram-negative in nature and aerobic. The morphological characteristics of the isolate are given in Table 1.

Biochemical characterization

The biochemical characterization of the isolated strain shows the positive results for catalase, oxidase, nitrate reduction to nitrites and urease (Table 2). The isolate can utilize galactose, glucose, fructose, mannitol, lactose and sucrose. The strain shows negative results for H_2S production, starch hydrolysis, motility and citrate utilization. The results show no utilization of mannose, xylose, rhamnose, raffinose and maltose. The bacterial isolate was preliminarily identified as *Azotobacter* sp. based on morphological and biochemical features.

Molecular characterization

The isolate was identified as *Azotobacter beijerinckii* through molecular characterization using 16SrRNA at NCMR-NCCS, Pune. The phylogenetic tree of the bacterial strain has been given in Fig. 2.

Qualitative analysis of nitrogen-fixing activity

The growth of isolate on GNFM containing BTB agar medium showed a significant change in the color of colonies indicating the nitrogen-fixing activity of the bacterial isolate (Fig. 3). Almost similar result was found in previous studies (40-42).

Production of IAA

The isolate was able to produce IAA in the Jensen medium amended with tryptophan and developed a pink color when reagents were added (Fig. 3). The amount of IAA produced was found to be 27 µg/mL by the isolate after 7 days of incubation. The results are shown in Table 2. The production of IAA was reported by previous workers (35, 38, 43).

Studies reported that *Azotobacter* spp. produced 38.82 µg/mL IAA in a culture medium supplemented with Tryptophan at the rate of 5 mg/mL (39). IAA production by *Azotobacter* spp. in the range of 42.80-82.00 µg/mL was

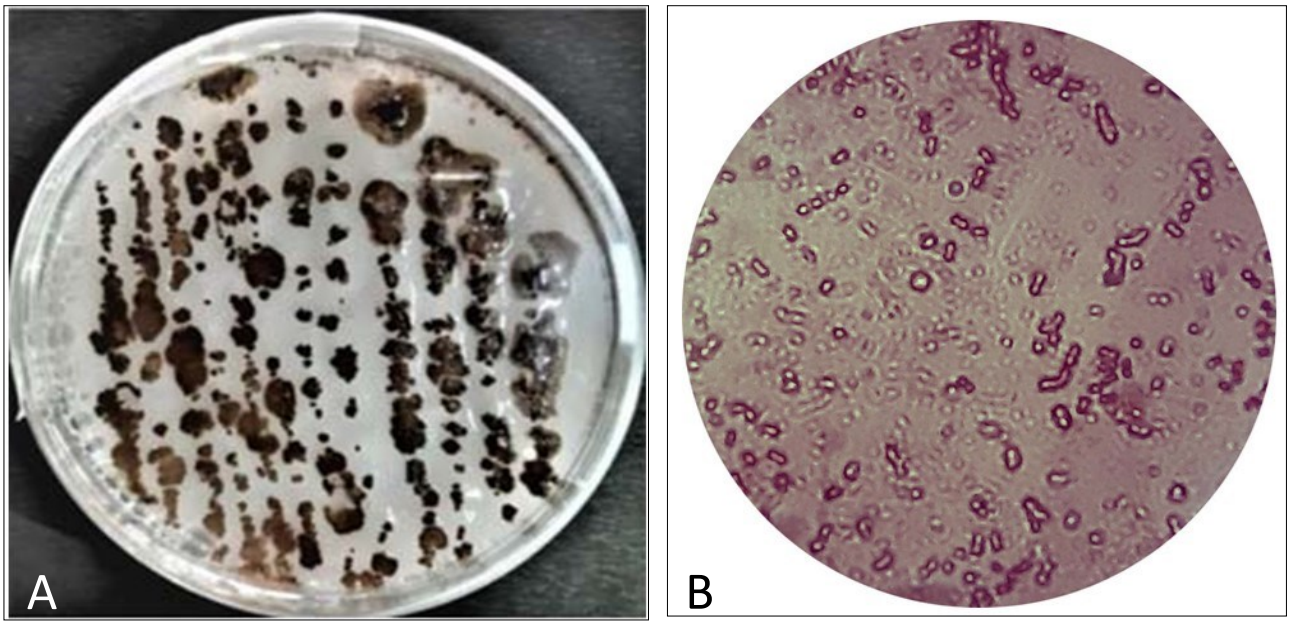

Fig. 1. Morphological characterization of strain TDH-01; (**A**) Plate showing pigmented colonies (**B**) Gram-stained cells of the bacteria under micro-

Table 1. Morphological characteristic of the isolated strain TDH-01.

Isolate	Gram nature	Shape	Colony Surface	Colony Margin	Colony Colour	Colony Texture	Pigment colour
TDH-01	-ve	Blunt Rod to ellipsoid	Smooth glistening	Entire	Off-white	Mucoid	Blackish

Table 2. Biochemical properties of the isolated strain TDH-01.

Biochemical characteristics	Result
Utilization of sugars	
Galactose	+
Mannose	-
Glucose	+
Fructose	+
Xylose	-
Mannitol	+
Rhamnose	-
Lactose	+
Sucrose	+
Raffinose	-
Maltose	-
Indole	+
Citrate	-
Catalase	+
Urease	+
Oxidase	+
H_2S production	-
NO_2 reduction	+
Motility	-
Starch hydrolysis	-

respectively.

Production of Gibberellic acid

The bacterial isolate was able to produce gibberellic acid (Fig. 3). The production of Gibberellic acid was found to be about 48 µg/mL. It was reported the production of Gibberellic acid by the *Azotobacter* sp. ranging between 02 µg/mL to 62 µg/mL (48). The production of Gibberellic acid by *Azotobacter* has been previously reported by many workers (49-51).

Table 3. Summarizes the values of phosphorus solubilized (µg/mL) and the change in pH of the corresponding medium, *in-vitro* production of IAA and Gibberellic acid after 7 and 3 days respectively.

Screening of siderophore production

The bacterial isolate produces a clear halo around the bacterial colony on the Chrome azurol S (CAS) agar medium indicating the production of siderophore (Fig. 3). A similar finding has been reported in earlier studies (52, 53).

Hydrogen cyanide (HCN) production

There was the development of orange to brown color in the Whatman filter paper that was impregnated at the inner surface of the lid of petri plates. The development of the orange color indicated that the bacterial isolate can

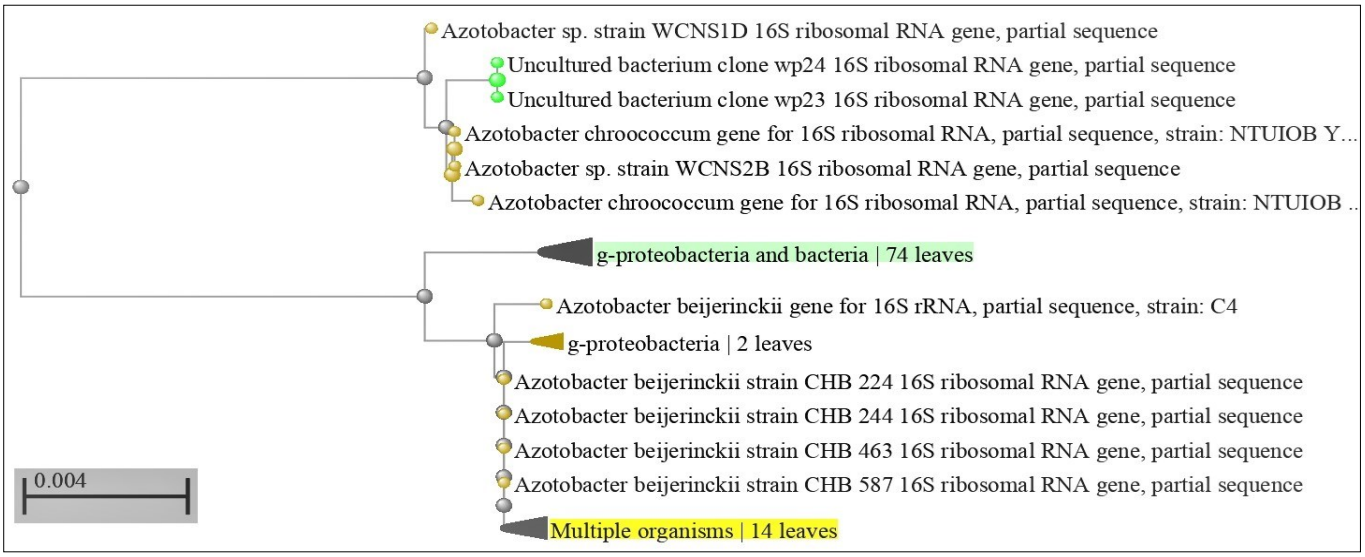

Fig. 2. Phylogenetic relation of the strain TDH-01 using the neighbour-joining method.

reported previously (44). Several other studies have also reported the IAA production in *Azotobacter* isolates (12, 45 -47).

Solubilization of Phosphate

The bacterial isolate was able to solubilize phosphate to a significant level as indicated by both methods of agar spot and quantification of phosphate supplemented with tricalcium phosphate in PKV broth (Fig. 3). The SI and SE of the bacterial isolate were found to be about 2.86 and 185

produce HCN (Fig. 3). Production of HCN by *Azotobacter* sp. was also reported in earlier studies (54).

Ammonia production

Bacterial isolate grown on peptone water shows the production of an orange color with the addition of Nessler's reagent which indicated the positive result for ammonia production (Fig. 3). A similar finding was reported in earlier study (55).

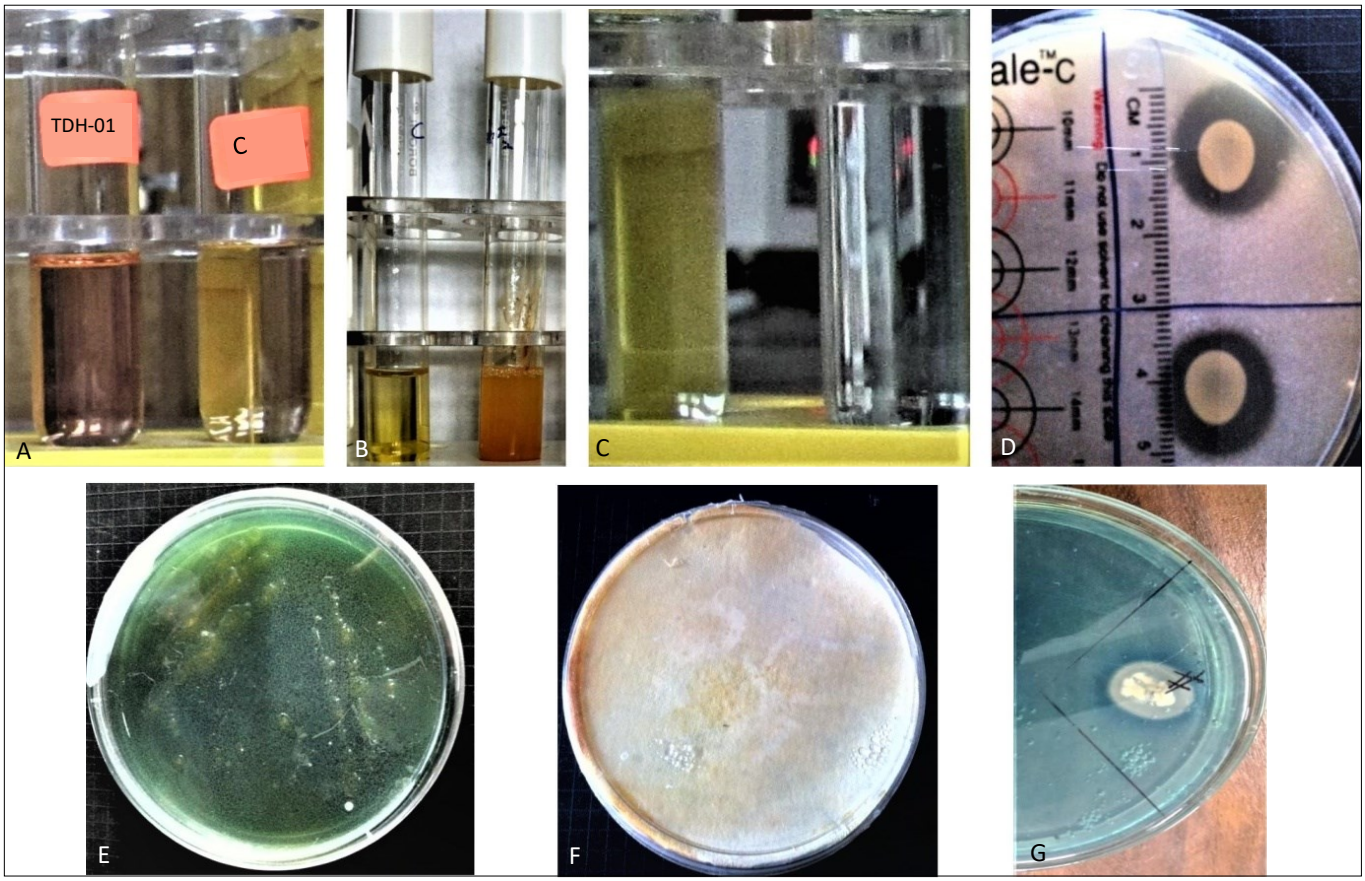

Fig. 3. Assessment of PGP characterization of strain TDH-01. (**A**) Positive and control sample for IAA activity, (**B**) Positive and control sample for Ammonia Production, (**C**) Control and positive sample from colorimetric estimation of Gibberellic acid production, (**D**) Clear halo zone around the colonies showing Phosphate solubilization, (**E**) Plate showing color changes of the colony for Nitrogen activity, (**F**) HCN production by bacterial isolates, (**G**) Clear zone around the colonies showing siderophore production.

Table 3. *In vitro* phosphate solubilisation, IAA production and Gibberellic acid production by the strain TDH-01.

Bacterial strain	pH of the medium	Available phosphorus (µg/ml) after 7 days of incubation	IAA production (µg/ml) after 7 days of incubation in presence of tryptophan	Gibberellic acid(µg/ml) production after 3 days of incubation
TDH-01	5.5	25	27	48

Table 4. Characterization of plant growth promoting traits of the isolated strain TDH-01.

Isolate	Nitrogen fixing activity	Siderophore producing ability	Hydrogen cyanide producing activity	Ammonia producing activity
TDH-01	Positive	Positive	Positive	Positive

Table 4. Summarizes the characteristic features of plant growth-promoting traits of the bacterial isolate.

In-vitro plant growth promotion test on Rice seeds using TDH-01 strain

The treatment of rice seeds with TDH-01 strain was found to be beneficial. % increase in germination over control was found to be 7.52% (Fig. 4 and Fig. 5). The strain showed improved seed germination and has a beneficiary response on the growth of shoot and root length (Fig. 6

Fig. 4. *In vitro* effect of strain TDH-01 on seed germination and vigor index. (**A**) Control and bacterial inoculated seed germination (**B**) Effect of bacterial inoculation on the growth of root and shoot of *Oryza sativa* seeds.

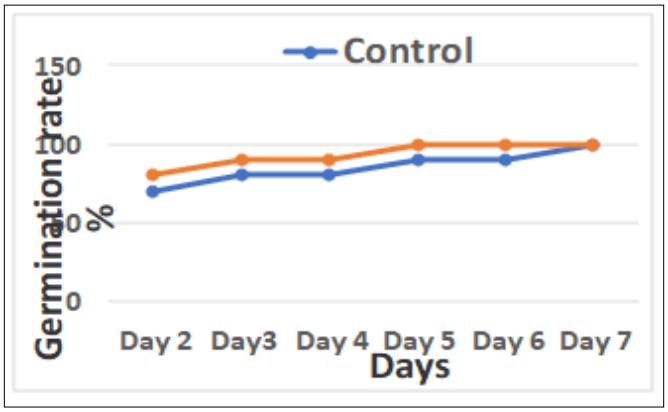

Fig. 5. Percentage of seed germination of control and treated seeds for 7 days.

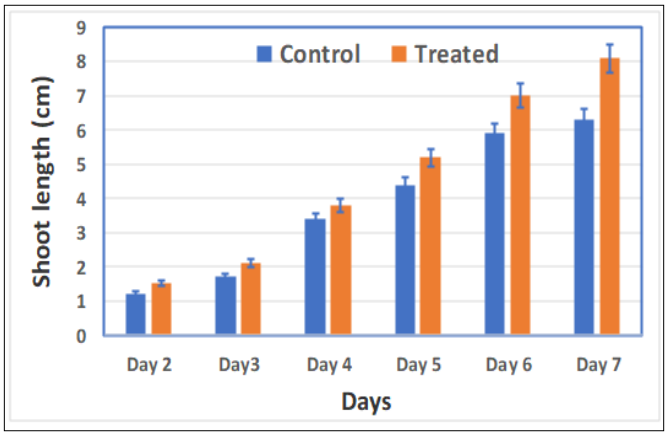

Fig. 6. Assessment of average shoot length of *Oryza sativa* for 7 days.

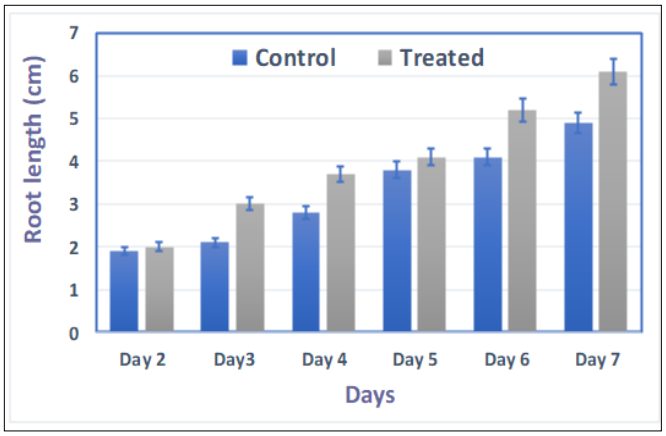

Fig. 7. Assessment of average root length of *Oryza sativa* for 7 days.

and Fig. 7). The vigor index calculated was found to increase in the treated seed in comparison to the control (Fig. 8). The finding was also supported by the report (56)

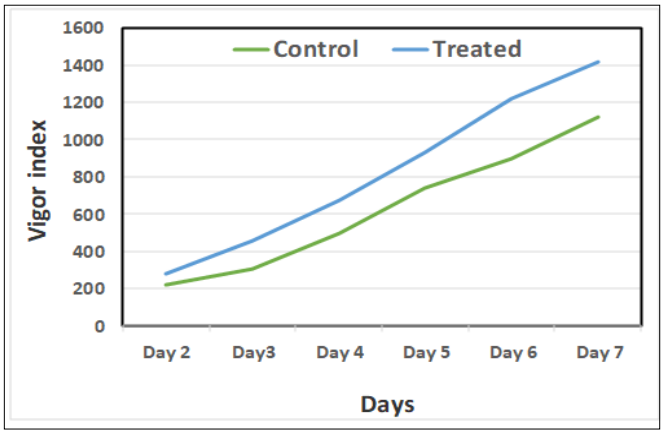

Fig. 8. Assessment of average Vigor index of *Oryza sativa* for 7 days.

that *Azotobacter* inoculation enhanced seed germination of rice, maize and wheat.

Greenhouse Experiment

In the greenhouse pot experiments, after 15 days of the plantation, the strain-treated seedling shows a significant increase in root length and shoot length than the control (Fig. 9). The results of the growth parameters of the treated and control are tabulated (Table 5). An increase in shoot length and root length by *Azotobacter* inoculation in the maize seedling has been reported in earlier studies (57).

Fig. 9. Greenhouse experiments (**A**) Control and bacterial inoculated rice seedling (left control) (**B**) Effect of inoculation of bacterial isolates on the growth of root and shoot of *Oryza sativa* seedling (extreme right control).

Table 5. Growth parameters of strain treated rice (*Oryza sativa)* seedling and percentage of increase over control.

Days after germination	Treatments	Shoot length (cm)	Root length (cm)
5	Control	3.86±0.54	1.07±0.39
	Treated with bacterial isolate	5.08±0.66	1.98±0.33
		31% [a]	45% [b]
10	Control	6.36±0.77	3.35±0.28
	Treated with bacterial isolate	8.18±1.06	4.38±0.40
		28% [a]	30% [b]
15	Control	8.74±1.13	4.44±0.70
	Treated with bacterial isolate	11.52±1.91	6.26±0.91
		31% [a]	40% [b]

a: % increase in shoot length with respect to control

b: % increase in root length with respect to control

Conclusion

It can be concluded from the above study that the strain TDH-01 isolated from the tomato rhizosphere of Darjeeling hills can produce plant growth-promoting substances like IAA, gibberellic acid and also can solubilize inorganic phosphate. The strains have the potential to be used as indigenous biofertilizers for the improvement of growth and yield of crops and hold substantial promise for advancing sustainable agricultural practices and environmental stewardship in the region. Through this study, significant insights into the plant-growth promotion (PGP) properties of the strains can be gained, potentially leading to the

development of novel bioinoculants tailored to enhance crop productivity in the unique agro-ecological context of Darjeeling hills.

The study not only contributes to the understanding of rhizosphere microbial diversity but also highlights the importance of harnessing indigenous microbial resources for agricultural improvement. By focussing on locally adapted microbial strains, the research has the potential to address specific challenges faced by farmers in Darjeeling hills, such as nutrient deficiency, soil degradation and sustainable resource management.

However, conducting research in this area presents challenges, including access to field sites, laboratory facilities and expertise. Collaborative partnerships, capacity-building initiatives and technology transfer efforts are crucial for overcoming these challenges and ensuring the success and sustainability of the research endeavors.

Overall, the isolation and characterization of *Azotobacter beijerinckii* for PGP properties from the tomato rhizosphere of Darjeeling hills represent a significant step towards the development of environmentally friendly and economically viable agricultural solutions tailored to the needs of the local farming community. By addressing these challenges and leveraging the region's microbial diversity, this research has the potential to contribute to food security, environmental conservation and sustainable development in Darjeeling Hills and beyond.

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Authors' contributions

Each author significantly contributed to the idea and design, data collection, analysis and interpretation. DB collected the data, carried out the isolation, biochemical and *in-vitro* PGP characterization and drafted the manuscript. SKC carried out the phylogenetic analysis. BCS carried out the statistical analysis. PCL designed the experimental work. All the authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: The authors express no conflict of interest.

Ethical issues: None.

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