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Isolation, identification and characterization of nutrient solubilizing bacteria (N fixer, P and K solubilizer) from the onion rhizosphere

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Abstract
Soil microorganisms play a vital role in nutrient cycling and plant growth by making essential nutrients like nitrogen, phosphorus and potassium bioavailable. This study aimed to isolate and characterize nitrogen fixing, phosphate and potassium solubilizing bacteria from the rhizosphere of onion plants in various districts of Maharashtra, India. Rhizospheric soil samples were collected and subjected to serial dilution and selective culturing using Jensen's, Pikovskaya's and Alexandrov's media to isolate *Azotobacter*, *Pseudomonas* and *Bacillus* species respectively. The isolates were purified, maintained and characterized through morphological, cultural, Gram staining and biochemical tests. Results revealed diverse colony morphologies and biochemical profiles, identifying probable species such as *Azotobacter* sp., *Pseudomonas* sp. and *Bacillus* sp. These beneficial microbes have potential application as biofertilizers, offering an ecofriendly alternative to chemical fertilizers and contributing to sustainable agricultural practices.

Keywords: *Biofertilizer*, *Azotobacter*, *Pseudomonas*, *Bacillus*

Introduction
Onion (*Allium cepa* L.) is a widely cultivated crop grown in diverse climates worldwide for over 5,000 years. It thrives in mild weather with optimal growth at 13-24 °C and bulb development at 16-25 °C under about 70% humidity. Being photoperiod sensitive, early and late varieties require 13 and 16 hours of daylight, respectively. In India, the rabi season accounts for around 70% of total onion production. Bio-fertilizers are increasingly recognized as a sustainable alternative to chemical fertilizers, enhancing soil fertility and plant nutrition through natural microbial processes. They include nitrogen fixing, phosphate and potassium releasing microorganisms that improve nutrient availability and soil health. Many also produce phytohormones and help plants resist stresses like drought, pests and diseases, supporting resilient and ecofriendly crop systems.
Soil microorganisms are vital for soil health and ecosystem balance, driving key nutrient cycles by making nitrogen, phosphorus and potassium available to plants. Nitrogen fixing bacteria (*Azotobacter*), phosphate solubilizing microbes (*Pseudomonas*) and potassium releasing bacteria (*Bacillus*) enhance plant nutrition and growth. Due to their efficiency and eco-friendliness, these microbes are widely used as biofertilizers, offering a sustainable alternative to chemical fertilizers.

Materials and Methods
Experimental site
The research work was conducted in the Department of Plant Pathology and Microbiology at Mahatma Phule Krishi Vidyapeeth, Rahuri which provided the necessary laboratory facilities, academic guidance and technical support essential for the successful execution of the study.

Collection of rhizospheric soil samples from onion rhizosphere
Rhizospheric soil samples were collected from onion growing villages in Pune, Ahilyanagar, Nashik and Dhule districts to isolate nitrogen fixing, phosphate and potash solubilizing bacteria. Samples were taken from 3 cm depth, sieved (4 mm mesh) and stored at field

moisture at 4 °C.

Isolation of rhizobacteria from rhizosphere soil

Isolation of Nitrogen fixing plant growth promoting rhizobacteria from rhizosphere soil

Nitrogen fixing bacteria were isolated from soil samples via serial dilution. 1 g of air dried soil was diluted up to 10^{-6} and 1 ml was plated on Jensen's medium, selective for nitrogen fixers. After 48 hours of incubation at 32 °C, bacterial strains were purified through repeated streaking and preserved as slant cultures. Identification was done using morphological and biochemical methods (Pal *et al*. 2017) [7].

Isolation of phosphate solubilizing rhizobacteria from rhizosphere soil

Phosphate solubilizing bacteria were isolated using Pikovskaya's medium following Aneja (2003) [1]. 10 g of rhizosphere soil were serially diluted (10^{-1} to 10^{-6}) and 1 mL from dilutions 10^{-3} to 10^{-5} was plated. Sterilized Pikovskaya's medium was added and plates were incubated at 28±2 °C for 4-5 days. Colonies forming clear zones indicated tricalcium phosphate solubilization. This procedure was repeated for all samples.

Isolation of potash solubilizing rhizobacteria from rhizosphere soil

Potassium solubilizing bacteria were isolated using the serial dilution plate method (Aneja, 2003) [1]. 10 g of rhizosphere soil was mixed with 90 mL sterile water, and 10^{-5} to 10^{-6} dilutions from rhizosphere suspensions were plated on Alexandrov medium containing mica. Plates were incubated at 30±1 °C for 3 days. Colonies with clear zones were selected and purified by four way streaking. The procedure was repeated for all samples, as also described by Hu *et al.* (2006) [4].

Purification, multiplication and maintenance of bacterial culture

A loopful of nitrogen fixing, phosphate and potassium solubilizing bacteria was streaked onto Jensen's, Pikovskaya's and Alexandrov's media respectively and incubated at 28 °C for colony development. Isolates were transferred to fresh plates to assess growth and measure inhibition zones. Purified cultures were maintained for further studies.

Characterization of pure isolates

Cultural and Morphological characterization of isolates

Microorganisms were identified based on cultural, morphological and biochemical characteristics following Bergey's Manual (2nd ed., 2009). Nitrogen fixing, phosphate and potassium solubilizing isolates were examined for cell shape, size, arrangement and motility. Overnight cultures were streaked on Jensen's, Pikovskaya's and Alexandrov's media and incubated at 28±2 °C for 48 hours to observe colony morphology.

Gram's staining

The isolate was characterized using the Gram staining procedure as outlined by Rosemary *et al*. (2013) [10]. A loopful of the culture was placed on a clean, dry slide to prepare a smear, which was air dried and heat-fixed. Two drops of crystal violet were applied and left for 30 seconds, followed by washing with distilled water. Next, 1-2 drops of Gram's iodine were added and left for 60 seconds, then the slide was washed with 95% ethyl alcohol. Safranin was added, left for 30 seconds and the slide was washed again with distilled water. After blotting to dry, the slide was observed under a microscope. Pink colonies indicated Gram-negative bacteria, while purple colonies indicated Gram-positive bacteria.

Biochemical characterization of isolates

The isolates were biochemically characterized using procedures described in the 10th edition of Microbiology: A Laboratory Manual. A series of tests including the Catalase test, Oxidase test, Indole production test, Methyl Red test, Voges-Proskauer (VP) test, Urea hydrolysis, Nitrate reduction test, Gelatin hydrolysis test, Starch hydrolysis, Casein hydrolysis, Siderophore production and H_2S production test was performed, following methodologies similar to those reported by Rosemary *et al.* (2013) [10], Upadhyay *et al.* (2015) [12] and Muthukumar *et al.* (2021) [6].

Catalase test

A small sample of the cultured organism was transferred to a labelled slide, followed by the addition of a drop of 3% hydrogen peroxide. The immediate formation of bubbles indicated a positive catalase test.

Oxidase test

Three drops of p-aminodimethylaniline oxalate were applied to the surface of the organism growth. The area was observed for a color change within 20-30 seconds. A positive oxidase test was indicated by the appearance of a purple color within 10-30 seconds, while the absence of color change denoted a negative result.

Indole production test

Using aseptic technique, the cultured organism was stab-inoculated into Tryptone broth in a deep tube. The tubes were then incubated at 37 °C for 24 to 48 hours. Following incubation, 10 drops of Kovac's reagent were added to each tube. The appearance of a red layer at the surface indicated a positive indole test, while the absence of a red layer indicated a negative reaction.

Nitrate reduction test

Using aseptic technique, the cultured organism was stab-inoculated into a deep tube of SIM agar and incubated at 37 °C for 24 to 48 hours. After incubation, 10 drops of Kovac's reagent were added. A red layer at the surface indicated a positive indole test, while the absence of a red layer indicated a negative reaction.

Urease test

Sterilized urea broth tubes were inoculated with overnight cultures and incubated at 50±2 °C for 48 hours. A color change to pink indicated a positive urease test, while no change in color was recorded as negative. A pre-sterilized, uninoculated urea broth served as the control.

H_2S test

The cultured organism was stab-inoculated into a labelled tube containing SIM agar medium and incubated at 37 °C for 24 to 48 hours. Ferrous ammonium sulfate in the medium served as an indicator for H_2S production, forming an insoluble black ferrous sulphide precipitate along the stab

line in the presence of hydrogen sulphide gas. The absence of black precipitate indicated a negative reaction.

Voges-Proskauer test

An MR-VP broth tube was inoculated with the test culture and incubated at 37 °C for 24 to 48 hours. Following incubation, 10 drops each of Barritt's reagent A and Barritt's reagent B were added sequentially. The development of a deep rose color within 15 minutes indicated a positive result for acetoin (acetylmethylcarbinol) production, while the absence of color change indicated a negative result.

Methyl red test

Third of the cultured organism was transferred to a labelled tube containing MR-VP broth and incubated at 37 °C for 24 to 48 hours. After incubation, five drops of methyl red indicator were added to the inoculated tube. A red color in the presence of the methyl red indicator, indicating a pH around 4, signified a positive result. A yellow color, indicating a pH of 6, was considered a negative result, as it still suggests acid presence but with a reduced hydrogen ion concentration.

Results and Discussion

Nitrogen fixing bacteria from rhizospheric soils were assessed for cultural and morphological traits to identify phenotypic variations and aid in preliminary classification, essential for selecting efficient strains for further studies and biofertilizer development (Aneja, 2003) [1]. All isolates were grown on Jensen's Agar at 28±2 °C for 48-72 hours, showing diverse colony morphology in size, shape, color, texture, elevation and margin. Pigmentation ranged from white to pale pink, with forms from circular to irregular and textures from smooth to wrinkled. Most colonies were raised or convex with entire or undulate margins (Table 01), consistent with the diversity reported by Wani *et al.* (2007) [14] among nitrogen-fixing bacteria from various agroecological zones. Gram staining showed most isolates were Gram negative rods, typical of Azotobacter, while some Gram-positive rods likely belonged to Bacillus. Several were motile, aiding root colonization (Verma *et al.* 2010) [13]. These findings highlight morphological diversity among free-living nitrogen fixers, aligning with Tilak *et al.* (2005) [11] and suggesting multiple agriculturally relevant genera.

Table 2 summarizes the cultural and morphological traits of phosphate solubilizing bacteria (PSB) isolates, all of which were motile, rod-shaped and showed pigmentation from white to yellow. Most formed circular colonies with entire or lobate margins and varied elevations. Gram staining revealed both Gram-positive and Gram-negative types. These characteristics align with findings by Wasule *et al.* (2007) [15], who reported similar colony shapes, textures and motility in PSB strains.

Tables 3 summarize the cultural and morphological traits of potash solubilizing bacterial isolates, all of which were motile, rod-shaped and mostly white or yellow pigmented. Colonies were circular with smooth margins and mostly raised elevations. Similar observations were reported by Panda *et al.* (2021) [8], who described diverse potash mobilizing bacteria as small, smooth, irregular, and including both Gram-positive rods and cocci.

The biochemical properties of pure bacterial isolates were analysed using standard tests to assess metabolic and enzymatic activities. These included Gram staining, starch hydrolysis, gelatin liquefaction, H_2S production, catalase, oxidase, and methyl red tests to evaluate cell structure, enzyme presence, and fermentation capabilities (Table 04). Patil *et al.* (2014) [9] isolated *Azotobacter chroococcum* from 12 of 22 soil samples using Jensen's medium. The gram-negative, rod-shaped isolates tested positive for most biochemical traits except the KOH test and showed optimal carbohydrate utilization with 1% sucrose salt. Positive PSB isolates were tested for catalase, oxidase, citrate utilization, urease, indole production, starch hydrolysis, and H_2S production using standard methods. Panda *et al.* (2021) [8] isolated 84 morphologically distinct rhizospheric colonies and screened them for potassium mobilization. Some were indole-negative, Voges-Proskauer-and citrate-positive, with mannitol motility. All showed positive results for esculin hydrolysis, catalase, oxidase, urease, and arginine dihydrolase.

Table 1: Cultural and Morphological Characterization of *Azotobacter* Spp.

Sr. No.	Isolates Name	Cultural Characterization				Morphological Characterization	
		Growth	Elevation	Appearance	Pigmentation	Shapes of Bacterial cell	Bacterial Cells arrangement
1	DSOA-01	Slight	Raised	Milky	Pale brown	Rods	Single cell
2	DSOA-02	Slight	Convex	Mucoid	Grey Brown	Rods	Single cell
3	DSOA-03	Abundant	Flat	Slimy	Light Brown	Rods	Cells are in pairs
4	DSOA-04	Abundant	Convex	Slimy	Pale brown	Oval	Cells are in pairs
5	NSOA-05	Abundant	Flat	Milky	Brown	Rods	Cells are in pairs
6	NSOA-06	Moderate	Raised	Mucoid	Pale brown	Rods	Cells are in pairs
7	NKOA-07	Moderate	Flat	Slimy	Light Brown	Rods	Cells are in pairs
8	NSOA-08	Slight	Raised	Milky	Pale brown	Oval	Cells are in pairs
9	NDOA-09	Moderate	Convex	Opaque	Grey Brown	Oval	Cells are in pairs
10	PIOA-10	Slight	Convex	Milky	Grey Brown	Oval	Single cell

Table 2: Cultural and Morphological Characterization of PSB isolates

Sr. No.	Isolates	Cultural Characterization			Morphological Characterization		
		Margin	Elevation	Appearance	Cell Shapes	Cell arrangement	Cell Motility
1	DSOP-01	Entire	Flat	Creamy	Rods	Cells in chains	Motile
2	DSOP-02	Entire	Raised	Whitish	Rods	Single cells	Motile
3	DSOP-03	Lobate	Convex	Creamy	Rods	Cells in chains	Motile
4	NSOP-04	Lobate	Convex	Creamy	Rods	Cells in chains	Motile
5	NSOP-05	Entire	Raised	Whitish	Rods	Single cells	Motile
6	NSOP-06	Entire	Flat	Creamy	Rods	Cells in chains	Motile
7	NSOP-07	Lobate	Convex	Whitish	Rods	Single cells	Motile
8	NSOP-08	Lobate	Convex	Creamy	Rods	Cells in chains	Motile
9	NKOP-09	Lobate	Flat	Creamy	Rods	Cells in chains	Motile
10	AROP-10	Entire	Raised	Creamy	Rods	Single cells	Motile

Table 3: Cultural and Morphological Characterization of KSB isolates

Sr. No.	Isolates	Cultural Characterization			Morphological Characterization		
		Margin	Elevation	Colour	Cell Shapes	Form	Cell Motility
1	DSOK-01	Entire	Flat	Creamy	Rod	Circular	Motile
2	DSOK-02	Entire	Raised	Creamy	Coccus	Circular	Motile
3	NKOK-03	Lobate	Raised	Creamy	Coccus	Circular	Motile
4	NKOK-04	Lobate	Raised	Whitish	Coccus	Filamentous	Motile
5	NSOK-05	Entire	Raised	Whitish	Coccus	Circular	Motile
6	NSOK-06	Entire	Flat	Creamy	Rod	Filamentous	Motile
7	NDOK-07	Lobate	Flat	Creamy	Rod	Circular	Motile
8	NNOK-08	Lobate	Flat	Creamy	Coccus	Circular	Motile
9	AROK-09	Lobate	Flat	Creamy	Rod	Circular	Motile
10	PBOK-10	Entire	Raised	Creamy	Coccus	Circular	Motile

Table 4: Biochemical characterization of Nitrogen fixer, PSB and KSB isolates

Sr. No.	Isolates Name	Gram Staining	Starch Hydrolysis	Gelatin Liquification	H_2S Production	Catalase Test	Oxidate Test	Methyl Red Test	Probable bacteria
1	NSOA-05	-	+	+	+	+	+	-	*Azotobacter Chroccocum*
2	NKOP-09	-	+	+	-	+	-	+	*Pseudomonas fluorescens*
3	DSOK-01	+	+	+	+	+	-	+	*Bacillus subtilis*

Conclusion

The study successfully isolated and characterized a diverse group of rhizobacteria namely nitrogen fixing, phosphate solubilizing and potash solubilizing bacteria from onion rhizospheric soils collected across four districts in Maharashtra. Using selective media and standard microbiological techniques, distinct bacterial colonies were obtained, purified and maintained for further study. Cultural, morphological and biochemical characterizations revealed a wide range of phenotypic diversity among the isolates. Most nitrogen fixing bacteria were Gram-negative rods (predominantly *Azotobacter* spp.), phosphate solubilizers were motile rods with variable Gram reactions (similar to *Pseudomonas fluorescens*) and potash solubilizers included both rods and cocci, with some identified as *Bacillus subtilis*. The observed variation in colony morphology, pigmentation, motility and biochemical traits reflects the presence of multiple efficient rhizobacterial strains with potential agronomic significance. These findings support earlier reports and underline the importance of rhizobacteria in sustainable agriculture. The characterized strains offer promise for development into biofertilizer formulations aimed at enhancing soil fertility and promoting plant growth in onion cultivation.

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