IJNRD.ORG ISSN : 2456-4184
INTERNATIONAL JOURNAL OF NOVEL RESEARCH AND DEVELOPMENT (IJNRD) | IJNRD.ORG
An International Open Access, Peer-reviewed, Refereed Journal

Isolation and Characterization of Culturable Phosphate-Solubilizing Rhizospheric and Endophytic Bacteria Associated with Cicer arietinum

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Abstract

This study explores the isolation and characterization of culturable phosphate-solubilizing bacteria (PSB) from rhizospheric soil and root tissues of Cicer arietinum in the Rewa district of Madhya Pradesh. From 36 soil and multiple root samples, 62 rhizospheric and 15 endophytic PSB isolates were obtained, with 11 strains showing notable drought tolerance. These strains exhibited key plant growth-promoting traits including phosphate and zinc solubilization, EPS and IAA production, HCN and ammonia release, siderophore synthesis, and ACC deaminase activity. Among them, strains MA1AA and SB5A stood out for their strong multifunctional potential under stress. These findings highlight their promise as biofertilizers to enhance chickpea growth under phosphorus-deficient and drought-prone conditions.

Keywords:

Phosphate-solubilizing bacteria (PSB), Cicer arietinum, Rhizospheric bacteria, Endophytic bacteria, Drought tolerance, Biofertilizer, Plant growth-promoting rhizobacteria (PGPR), EPS, IAA, Zinc solubilization, ACC deaminase, Rewa district.

1. Introduction

Phosphorus (P) is a fundamental macronutrient required for plant growth and development, playing a central role in numerous physiological and biochemical processes, including energy transfer, signal transduction, photosynthesis, and the synthesis of nucleic acids and membrane phospholipids (Sharma et al., 2013). Despite its importance, the bioavailability of phosphorus in agricultural soils is limited due to its fixation into insoluble forms such as tricalcium phosphate, iron phosphate, and aluminum phosphate, which are inaccessible to plants (Rodríguez & Fraga, 1999). To overcome phosphorus deficiency, conventional agriculture relies heavily on chemical fertilizers; however, their excessive use leads to soil degradation, reduced microbial diversity, eutrophication, and economic burden (Khan et al., 2009). In this context, phosphate-solubilizing bacteria (PSB) offer an eco-friendly and cost-effective alternative by mobilizing insoluble phosphate through the secretion of organic acids and phosphatases, thus improving soil fertility and plant productivity (Chen et al., 2006; Zaidi et al., 2009).

Chickpea (*Cicer arietinum* L.) is an important leguminous crop grown extensively in India and other semi-arid regions, valued for its high protein content and ability to enrich soil nitrogen through symbiosis with *Rhizobium* species (Gaur et al., 2012). However, chickpea cultivation is often constrained by abiotic stresses, especially drought, and phosphorus deficiency, which together impair plant growth and yield (Kumar et al., 2012). Drought stress, in particular, disrupts water uptake, nutrient absorption, and hormonal balance in plants, making it essential to explore sustainable biological solutions that can mitigate such stress conditions. In this regard, plant growth-promoting rhizobacteria (PGPR), including PSB, have shown promise not only in nutrient solubilization but also in enhancing drought tolerance through multiple mechanisms such as production of indole-3-acetic acid (IAA), exopolysaccharides (EPS), siderophores, ammonia, hydrogen cyanide (HCN), and the enzyme ACC deaminase (Glick, 2014).

Rhizospheric and endophytic bacteria, which inhabit the root surface and internal plant tissues respectively, play pivotal roles in improving nutrient uptake, stress tolerance, and plant growth regulation. Endophytes are particularly advantageous as they colonize internal plant niches and maintain a stable association, even under harsh environmental conditions (Ryan et al., 2008). The identification of such stress-tolerant and multifunctional PSB from chickpea grown in drought-prone regions like the Rewa district of Madhya Pradesh can serve as a valuable resource for the development of location-specific bioinoculants.

2. Methodology

This study aimed to isolate, characterize, and evaluate plant growth-promoting attributes of drought-tolerant rhizobacteria associated with *Cicer arietinum* (chickpea). The methodology involved soil sampling, isolation of rhizobacterial isolates, morphological and biochemical characterization, drought tolerance screening, and evaluation of plant growth-promoting traits such as IAA production, phosphate and zinc solubilization. (Cappuccino JG et al.2010)

Isolation of Rhizobacteria

Soil samples were collected from chickpea rhizospheres in drought-prone regions of Madhya Pradesh. A 10^{-1} dilution was prepared, and bacterial cultures were plated on nutrient agar (NA). Distinct colonies were sub-cultured for purification and preservation at 4°C. (Schwyn B et al. 1987)

Morphological and Biochemical Characterization

Isolates were characterized based on colony features and subjected to Gram staining and biochemical tests (indole production, MR, VP, catalase, oxidase, citrate utilization, TSI, and gelatin liquefaction). (Penrose DM et al.2003)

Screening for Drought Tolerance

Isolates were cultured on nutrient agar with polyethylene glycol (PEG 6000) at various concentrations to simulate drought conditions. The isolates' ability to grow at higher PEG concentrations indicated drought tolerance. (Sambrook J et al.2001)

Evaluation of Plant Growth-Promoting Traits

- **IAA Production:** Measured using the Salkowski reagent method with LB broth supplemented with L-tryptophan.
- **Phosphate Solubilization:** Tested on Pikovskaya's agar medium, with the solubilization index (SI) calculated.
- **Zinc Solubilization:** Evaluated using modified Tris minimal agar medium with insoluble zinc compounds, and SI was calculated similarly.

Cyanide (HCN) Production

HCN production was assessed by growing isolates on Luria agar with glycine. A color change on filter paper soaked in sodium carbonate and picric acid indicated HCN production.

Ammonia Production

Ammonia production was quantified using Nessler's reagent in peptone water medium. The color change indicated ammonia levels, and optical density was measured at 635 nm.

Siderophore Production

Siderophore production was tested on Chrome Azurol S (CAS) agar. The formation of an orange halo indicated siderophore production, and SI was calculated.

ACC Deaminase Activity

ACC deaminase activity was screened by growing isolates in modified DF minimal medium supplemented with ACC. The ability to utilize ACC as a nitrogen source confirmed ACC deaminase activity.

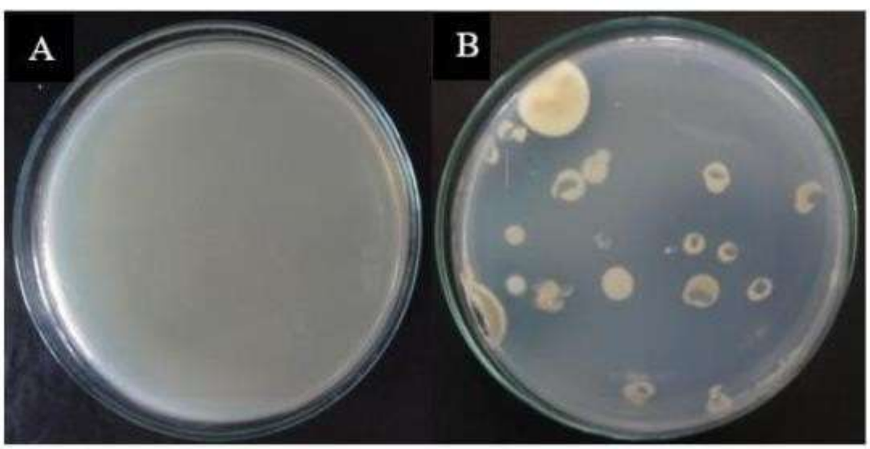
DNA Extraction and Genetic Identification

DNA was extracted, and the 16S rRNA gene was amplified by PCR. Sequenced PCR products were analyzed using BLAST for species identification, and phylogenetic analysis was conducted to study evolutionary relationships. (Altschul SF et al. 1990)

3. Result and Discussion

Isolation of Culturable Phosphate-Solubilizing Rhizospheric and Endophytic Bacteria

A total of thirty-six rhizospheric soil samples were collected from twelve distinct locations across the Rewa district, Madhya Pradesh, India, to isolate phosphate-solubilizing bacteria (PSB). Soil samples were serially diluted and cultured on nutrient agar medium (NAM) under sterile conditions. Following incubation, bacterial colonies were isolated, and colony-forming units (CFU) were quantified, with an average population density of 1×10^5 CFU/g of soil recorded.



A=Control plate, B= Rhizospheric bacteria showing clear halo zones on PAM

Fig.1. Isolation of culturable phosphate solubilizing rhizospheric and endophytic bacteria.

All bacterial isolates were screened for phosphate-solubilizing ability using phosphate agar medium (PAM). Colonies exhibiting a distinct halo zone, indicative of phosphate solubilization, were identified as potential PSB. From the rhizospheric samples, a total of 80 PSB isolates demonstrating halo formation were selected. These representative isolates were further purified and stored at –80°C in glycerol stocks for future studies.

In parallel, endophytic PSB were isolated from root tissues of *Cicer arietinum*. Roots were thoroughly washed with sterile distilled water, surface sterilized sequentially using 95% ethanol and sodium hypochlorite, and then rinsed in sterile water. The sterilized roots were macerated in phosphate-buffered saline (PBS), and the suspension was centrifuged to obtain bacterial pellets. These pellets were plated onto NAM and incubated at 37°C for 24 hours.

Emerging colonies were subsequently transferred onto PAM and incubated at 30°C for 48 hours to assess phosphate solubilization. A total of 15 endophytic PSB isolates were identified based on the presence of clear halo zones on PAM.

Confirmation of Phosphate-Solubilizing Ability

The phosphate-solubilizing activity of the bacterial isolates was confirmed by the presence of clear halo zones surrounding the colonies on PAM plates, indicating the release of soluble phosphate. However, it was observed that a subset of isolates initially identified as PSB lost their solubilization potential upon subsequent culturing. Out of the 80 rhizospheric PSB isolates, only 62 retained consistent halo formation and were selected for further characterization and downstream analysis.

Purification and Morphological Characterization of PSB Isolates

To ensure the purity of the confirmed phosphate-solubilizing bacterial (PSB) strains, all 62 isolates were re-streaked on fresh nutrient agar medium (NAM) plates using the quadrant streaking method. After incubation, colonies were examined for uniformity in morphology to confirm purity.

The purified isolates exhibited diverse colony morphologies. Observed characteristics included smooth and irregular colony edges, with surface textures ranging from creamy to dry. Colony pigmentation varied among isolates, displaying colors such as yellow, pale yellow, cream, and off-white. Based on these phenotypic traits, the isolates were preliminarily classified into distinct morphological groups and stored at –80°C in glycerol stocks for subsequent physiological, biochemical, and molecular characterization.

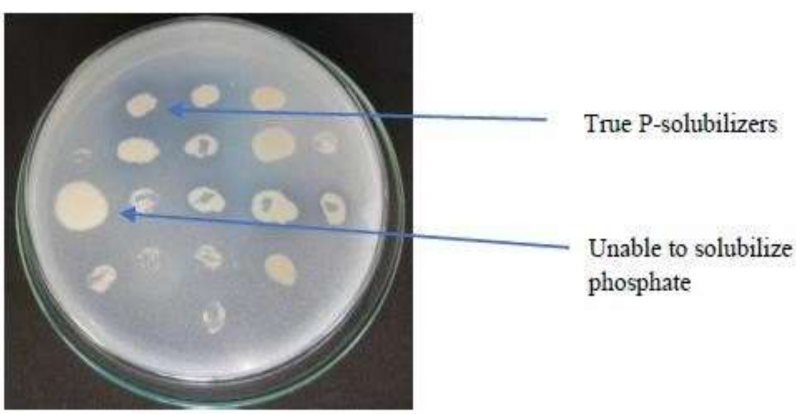


Fig.2. Reconfirmation of PSB on Pikovskaya's agar medium.

Morphological and Biochemical Characterization of PSB Isolates

The 62 purified phosphate-solubilizing bacterial (PSB) isolates were subjected to morphological and biochemical characterization to assess their taxonomic and functional diversity. Colony morphology varied significantly among the isolates, ranging from smooth, creamy, off-white colonies to irregular, mucoid, and filamentous textures. These variations suggest a wide range of bacterial genera contributing to phosphate solubilization activity.

Biochemical assays revealed that the majority of the isolates were Gram-negative, catalase-positive, and oxidase-negative. These traits are commonly associated with phosphate-solubilizing rhizobacteria and reflect their metabolic adaptability to different soil environments.

Drought Tolerance of PSB Isolates

The drought tolerance of 62 PSB isolates from *Cicer arietinum* rhizospheric soil was assessed using polyethylene glycol (PEG 6000) to simulate osmotic stress. Eleven isolates (MA1AA, RG1A, CL3A, CL3B, RH2B, SB4B, BK2C, BS2AA, BT3AA, SB5A, and DT4B) demonstrated notable growth under PEG concentrations ranging from 10% to 50%, indicating drought resilience.

Survival was further evaluated in Trypticase Soy Broth with varying PEG levels. MA1AA showed the highest tolerance, surviving up to 50% PEG, while DT4B survived only up to 20%. These drought-tolerant strains may serve as potential bioinoculants for stress-prone environments.

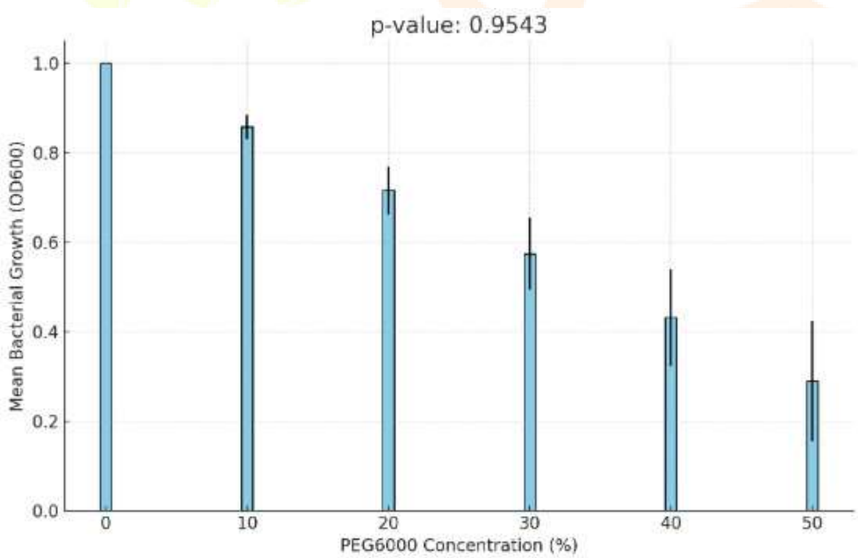


Fig.3. Growth of eleven rhizobacteria under non-stressed (control) and drought-stressed conditions at different PEG concentrations.

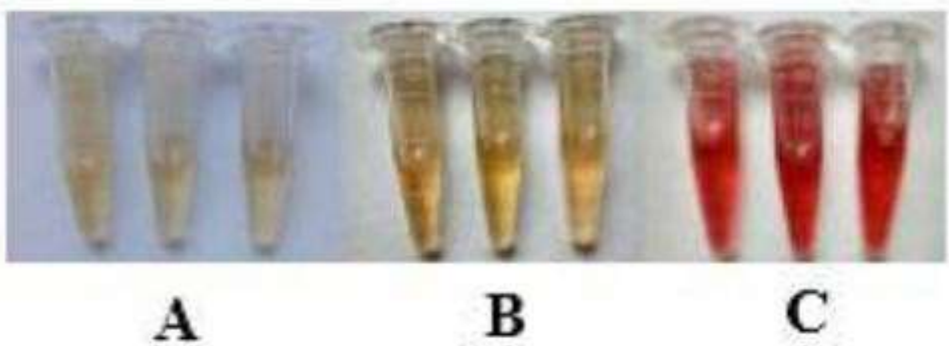
Characterization of Rhizobacteria for Plant Growth-Promoting Traits

Exopolysaccharide (EPS) Production:

EPS production was assessed in 11 drought-tolerant bacterial isolates under both normal and drought (20% PEG) conditions. Strain MA1AA exhibited the highest EPS yield in both conditions, followed by CL3B, SB4B, and SB5A. EPS plays a crucial role in improving soil structure, conserving moisture, and aiding bacterial colonization of plant roots. Its presence also contributes to stress tolerance and biofilm formation, making EPS-producing strains valuable for drought-prone agricultural systems.

Indole-3-Acetic Acid (IAA) Production:

IAA production was evaluated using the Salkowski reagent, both with and without L-tryptophan. None of the isolates produced IAA in the absence of tryptophan, but all produced measurable quantities when supplemented, with SB5A (41.87 µg/ml) leading, followed by CL3A and RH2B. IAA promotes root elongation and branching, improving nutrient and water absorption. These results suggest that these isolates could significantly enhance plant root development under stress.

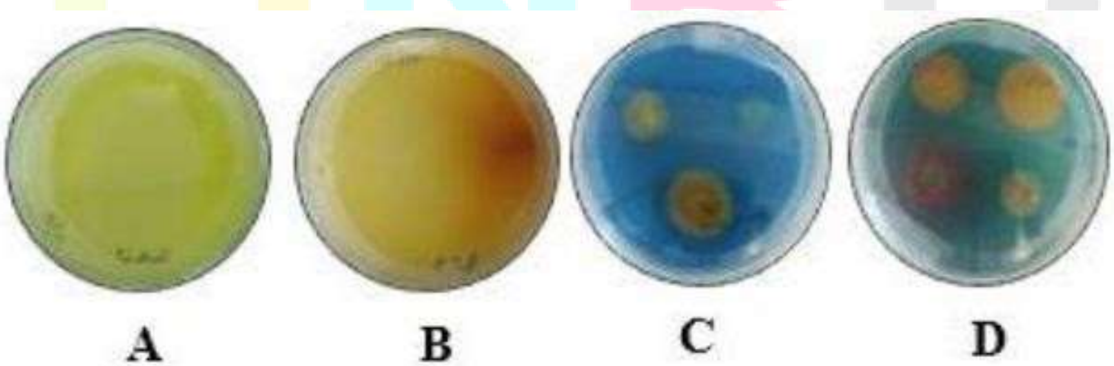


A=Control, B= IAA production without addition of tryptophan, C = IAA production with addition of 200 µg/ml tryptophan.

Fig.4. IAA production shown by bacterial strains.

Phosphate Solubilization:

All 62 bacterial isolates showed clear halo zones on Pikovskaya's agar medium, indicating their ability to solubilize inorganic phosphate. This is an essential trait for improving phosphorus availability in soils, where most phosphate exists in insoluble forms. Efficient phosphate solubilization helps promote plant growth, reduce chemical fertilizer dependence, and enhance nutrient use efficiency. These PSB strains show promise as biofertilizers for sustainable agriculture.



A=*E.coli* as negative control, B=Orange color production of HCN by bacterial strain;

C, D=Siderophore production

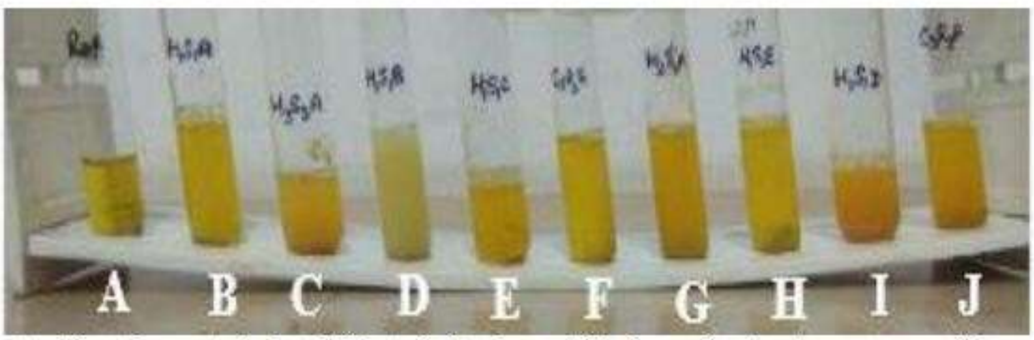
Fig.5. HCN production by bacteria.

Zinc Solubilization:

The ability to solubilize insoluble zinc compounds was tested in 11 isolates, all of which showed positive results. Formation of clear halos on nutrient agar supplemented with zinc indicated the production of organic acids or chelators. Zinc solubilization is vital for supporting plant enzyme function and hormone regulation. These isolates may be useful in improving zinc nutrition in zinc-deficient soils.

Hydrogen Cyanide (HCN) Production:

HCN production was confirmed in eight out of eleven isolates using picric acid-impregnated filter paper. Strains such as RH2B, MA1AA, and SB5A showed strong HCN activity. This trait is significant for biological control of soil-borne pathogens, as HCN inhibits harmful microbial activity in the rhizosphere. The HCN-producing strains have potential application in integrated disease management strategies.



A= Negative control, B to J=Bacterial strains, which showed color change as a positive indication for ammonia production

Fig.6. Ammonia production shown by bacterial strains.

Ammonia Production:

All 11 bacterial isolates tested positive for ammonia production, as indicated by a color change upon the addition of Nessler's reagent. Ammonia is an important nitrogen source and enhances soil fertility by contributing to plant nitrogen uptake. This trait is beneficial for reducing chemical fertilizer inputs while promoting plant protein and chlorophyll synthesis. Ammonia-producing PGPR strains are valuable for eco-friendly agricultural practices.

Siderophore Production:

Siderophore production was evaluated using chrome azurol S (CAS) agar, with seven isolates showing orange halo zones. Strain SB4B exhibited the largest zone (16 mm), followed by SB5A and BT3AA. Siderophores improve iron acquisition, especially in iron-deficient soils, supporting chlorophyll formation and plant metabolism. Their role in limiting pathogen growth through iron **competition further enhances their biocontrol potential.**

A B C D

A=Control plate of *Rosellinia* sp., B=Test plate; C= Control plate of *Fusarium* sp., D= Test plate

Fig.7. Siderophore production by bacteria.

ACC Deaminase Activity:

ACC deaminase activity was observed in four isolates (MA1AA, CL3B, SB4B, and SB5A), indicated by growth on minimal media with ACC as the sole nitrogen source. This enzyme reduces plant ethylene levels, which are elevated under stress conditions such as drought or salinity. By lowering ethylene, these bacteria support better root development and plant growth under stress. ACC deaminase-positive strains are particularly valuable for stress-tolerant bioinoculant development.

(A) (B) (C)

(A) Growth of isolates on NAM, (B) Growth of isolates on DF minimal medium with nitrogen source (positive control), (C) Growth of isolates on DF minimal medium with ACC as nitrogen source.

Fig.8. Screening of bacterial isolates for 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity.

5. Conclusion

This research highlights the intricate relationship between soil health and microbial biodiversity, emphasizing the pivotal role of phosphate-solubilizing bacteria (PSB) in enhancing agricultural productivity. Comprehensive physico-chemical analysis of soils from four districts in Madhya Pradesh revealed a wide range of pH, EC, organic carbon, and nutrient levels, underscoring spatial variability in soil fertility. The successful isolation of 80 PSB strains, including 62 stable and active phosphate-solubilizers, from both rhizospheric and endophytic environments, demonstrates the rich microbial potential within Cicer arietinum-associated soils. These isolates, characterized by diverse colony morphologies and biochemical traits, exhibit promising capabilities for biofertilizer development.

The study reinforces the importance of site-specific microbial profiling and paves the way for future molecular and field investigations to integrate native PSBs into sustainable farming systems, reducing chemical input and fostering long-term soil health.

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