RESEARCH ARTICLE

ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF HALOTOLERANT PHOSPHATE-SOLUBILIZING BACTERIA (PSB) FOR BIOFERTILIZER PRODUCTION

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Abstract:

Halotolerant phosphate-solubilizing bacteria (PSB) contribute significantly to enhancing phosphorus availability in salt-contaminated soils, promoting crop yield, and decreasing reliance on chemical fertilizers. Herein, nursery soil samples yielded halotolerant PSB using serial dilution and spread plating on Pikovskaya agar amended with NaCl. Preliminary screening spotted phosphate-solubilizing strains based on halo zone appearance. The shortlisted isolates were subjected to biochemical characterization, enzymatic function evaluation, and halotolerance determination at different concentrations of NaCl. The best performing strains showed significant phosphate dissolving capability at high salinity, and they have the potential for use in PSB-based biofertilizer production. Field use of such strains shall enhance soil fertility, plant vigor, and sustainability of salt-contaminated agricultural fields.

Keyword: Halotolerant PSB, Phosphate Solubilization, Salinity, Biofertilizer, Sustainable Agriculture.

Introduction:

Phosphorus is the second most critical plant macronutrient following nitrogen and is an integral component of nucleic acid production, cell division, and construction of new tissues and plays a role in photosynthesis, carbohydrate metabolism, energy production, redox homeostasis, and plant signalling [1]. Despite its importance, the majority of phosphorus in soil exists in an insoluble state making it

unavailable to plants and necessitating the excessive use of chemical fertilizers leading to environmental issues such as eutrophication and soil degradation [2]. Phosphate-solubilizing microorganisms (PSM), among the plant growth-promoting rhizobacteria (PGPR), have the potential to convert the insoluble phosphorus into plant-accessible forms and thereby ensure soil nutrient equilibrium. Some of these include phosphate-solubilizing bacteria (PSB) and actinomycetes as well as these often function synergistically with arbuscular mycorrhizal fungi [3]. PSB have, for the most part, been categorized into organic P-mineralizing bacteria that mineralize organic phosphorus substrates and inorganic phosphate-solubilizing bacteria that convert insoluble inorganic phosphate substrates into solution [4]. PSB play a role in the soil phosphorus cycle by dissolving–precipitating, mineralizing–fixing, and adsorption–desorption processes and these mostly occur by means of organic acids and phosphatases and other enzyme secretions by PSB to demineralize phosphate or to chelate cations like Ca^{2+}, Fe^{2+}, and Al^{3+} and provide phosphate ions [5]. PSB also impact soil microbial richness and enzymatic activities and enhance crop growth and yield [6]. Salinity, a significant abiotic factor impacting crop production, decreases water absorption, ion toxicity, reduces transpiration, and increases leaf senescence [7] and, in addition, disrupts synthesis of proteins, lipid recycling, and photosynthesis. The function of PSB in salt soils has been less studied; nonetheless, halotolerant PSB have the potential to sustain phosphate solubilizing function in the face of osmotic stress and have a potential sustainable solution for enhancing phosphorus availability in salt-contaminated soils [8]. The bacteria exhibit various plant growth-promotion characteristics, such as organic acid production and enzymatic function, and have usually been isolated by primary screening on Pikovskaya agar and subsequent characterization of their biochemistry, sugar use, enzyme determination, and mobility analysis [9]. The use of efficacious halotolerant PSB within carrier-based biofertilizers has the potential to increase phosphorus availability, enhance soil fertility and microbial numbers, increase crop yield under salt conditions, and ensure less usage of chemical fertilizers [10]. The current work focuses on the isolation, identification, and characterization of halotolerant PSB from salt soils for the production of biofertilizer products aimed at enhancing agricultural production in salt-affected areas.

Materials and Methods

Sample Collection and Preparation

Soil samples were collected in winter 2025 from horticultural nurseries at North Nanded and Tuppa regions of Maharashtra, India, and the latter was designated as the region of salt-affecting soil. The samples were subjected to air-drying and a process of sieving and further preserved in sterile containers for later analysis, following standardized practices of sample collections given by Jackson (1973) and Subba Rao (1999).

Isolation of Halotolerant Phosphate-Solubilizing Bacteria (PSB)

Two of the procedures used for isolation at different NaCl concentrations were by following the protocols of Pikovskaya (1948) and Nautiyal (1999) as modified. The first technique involved the use of 100 mL of Pikovskaya broth containing 5% NaCl and subsequent autoclaving. Aseptically, a

gram of soil was added and incubated at 37°C for 3–4 days for enrichment. Turbidity and pellet formation indicated the presence of bacteria. The enrich culture was serially dilutionned from 10^{-1} to 10^{-5} and 100 µL amounts from dilution 10^{-2} to 10^{-5} were plated on Pikovskaya agar and Modified $Ca_3(PO_4)_2$ agar [2].

In the second approach, 1 g of soil was suspended in 100 mL of sterile distilled NaCl solution containing 1% NaCl and subjected to serial dilution before plating on Pikovskaya agar. Colonies showing distinct halo zones indicating phosphate solubilization [11] were further purified and kept on LB agar slants at 4°C.

Primary Screening

These primary screenings involved spread plating on Pikovskaya agar supplemented with tricalcium phosphate [12]. PSB positives relied on halo zone formation surrounding the colonies, colonization at various NaCl concentrations (halotolerance test), and colony morphology analysis [3].

Secondary Screening

Secondary screening for functional characteristics:

- **Quantitative Solubilization of Phosphate:** Isolates were grown on PVK or NBRIP broth with added insoluble phosphate at 37°C, 120–150 rpm for 5–7 days and extractable phosphate was estimated by vanadomolybdate method [13] or Murphy & Riley's technique (1962).
- **pH Reduction**: Both initial and terminal pH of the broth were monitored to verify organic acid production [6].
- **Motility:** Motility was carried out by Soft agar stab test [14].
- **Carbon Source Utilization:** Carbon source utilization was established by virtue of oxidative-fermentative and carbohydrate fermentation tests with phenol red broth [15].

Biochemical and enzymic tests including catalase, amylase, gelatin liquefaction, protease, casein hydrolysis, citrate usage, methyl red test (MR), Voges–Proskauer test (VP), nitrate reduction, and Gram staining were performed using standard microbiological procedures [16].

Identification of Potential PSB Strains

Phenotypic characterization was done by means of biochemical and morphologic data. Identification was aided by the Online Identification of Bacteria and the ABIS Online, based on probability matrices for species prediction from metabolic characteristics [9].

Biofertilizer Production

Most efficient isolate (APP5) was mass-cultured on Pikovskaya broth under salt conditions and maintained at 37°C for 5 days. The culture was introduced with sterilized soil used as the carrier material for the development of a solid carrier-based biofertilizer, which was allowed to dry and stored, according to the Subba Rao's protocol for the manufacture of biofertilizers (1999).

Seed Germination Test

Seeds of wheat (*Triticum aestivum*), mung bean (*Vigna radiata*), moth bean (*Vigna aconitifolia*), peanut (*Arachis hypogaea*), pigeon pea (*Cajanus cajan*), chickpea (*Cicer arietinum*), brown chickpea (*Cicer arietinum*), and green pea (*Pisum sativum*) were analyzed following the procedures set by Khan *et al.* (2010). Two separate treatments were applied: Control: Seeds kept in

sterile distilled water for 30 minutes. Biofertilizer: Seeds kept in a solution of phosphorus-solubilizing bacteria (PSB) for 30 minutes.

Seeds were stored in sterile Petri plates with moist filter paper and kept at room temperature for 4 days. The germination percentage, the length of the primary and secondary roots and shoots, biomass, and phosphorus uptake were estimated.

Results and Discussion:

Isolation and Morphological Characterization of PSB

Soils contaminated with 5% NaCl were cultured on Pikovskaya agar and on Modified $Ca_3(PO_4)_2$ agar. The latter induced slower proliferation compared to PVK medium, as previously expected from reports which indicated that PVK favors better enrichment of phosphate-solubiling bacteria under salt conditions [17]. At 1% NaCl, enhanced proliferation and wider halo zones were observed (Figure 6), similar to the report of Illmer & Schinner (1995) on halo production as proof for phosphate solubilization.

Eight rapidly growing colonies (APP1–APP8) showing flat white to creamy, butyrous, and opaque characteristics were selected for further study (Table 1). Comparable colony appearances have been reported for Pseudomonas and Enterobacter plant growth-promoting bacteria in salt-containing soil conditions [18].

Table 1: Colony morphology of isolates

	APP1	**APP2**	**APP3**	**APP4**	**APP5**	**APP6**	**APP7**	**APP8**
Shape	Irregular	Irregular	Irregular	Irregular	Irregular	Irregular	Irregular	Irregular
Size (mm)	5 mm	4 mm	5 mm	3 mm	4 mm	5 mm	4 mm	3 mm
Margin	Entire	Entire	Entire	Entire	Entire	Entire	Entire	Entire
Surface	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Color	Creamy	Creamy	Creamy	Creamy	Creamy	Creamy	Creamy	Creamy
Consistency	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous
Elevation	Raised	Raised	Raised	Raised	Raised	Raised	Raised	Raised
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque

Screening and Biochemical Characterization

These eight isolates were Gram-negative rods (Figure 1; Table 2). Gram-negative dominance by halotolerant PSB is not rare, as revealed by Khan *et al.* (2009) and Mehta & Nautiyal (2001).

Table 2: Gram staining and motility of isolates

	APP1	**APP2**	**APP3**	**APP4**	**APP5**	**APP6**	**APP7**	**APP8**
Gram	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Motility	Motile	Motile	Motile	Motile	Motile	Motile	Motile	Motile

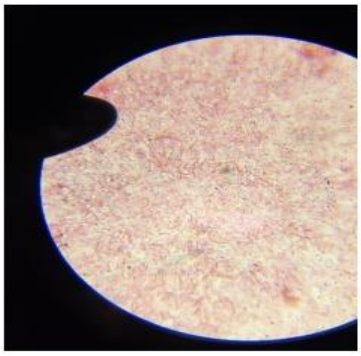


Figure 1: Microscopic gram staining slide (Gram-negative rod shape PSB bacteria)

Pattern of sugar consumption (Tables 3; Figure 2) showed usage of glucose and mannitol by all strains, and lactose fermentation varied. Gas production was not a common feature and was consistent with published PSB screening reports previously [6].

Table 3: Sugar tests

	APP1	APP2	APP3	APP4	APP5	APP6	APP7	APP8
Acid Production								
Glucose	+	+	+	+	+	+	+	+
Lactose	+	+	+	-	+	+	-	+
Mannitol	+	+	+	+	+	+	+	+
Gas Production								
Glucose	-	+	-	-	-	+	-	+
Lactose	-	-	-	-	-	-	-	-
Mannitol	-	-	-	-	-	-	-	-

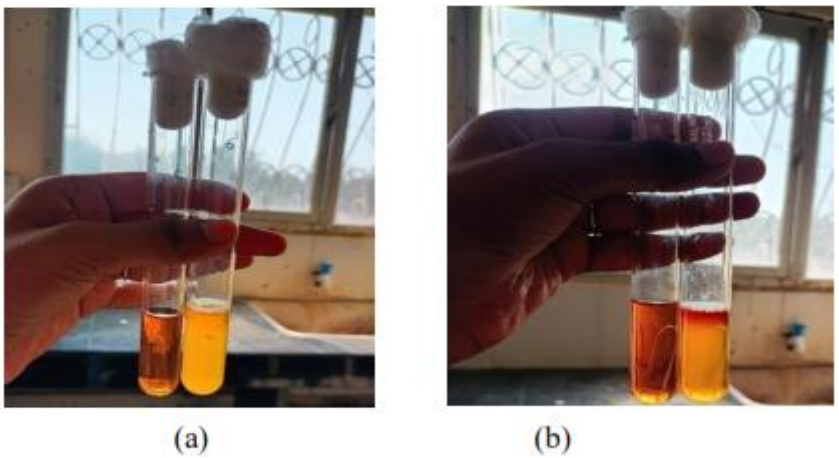
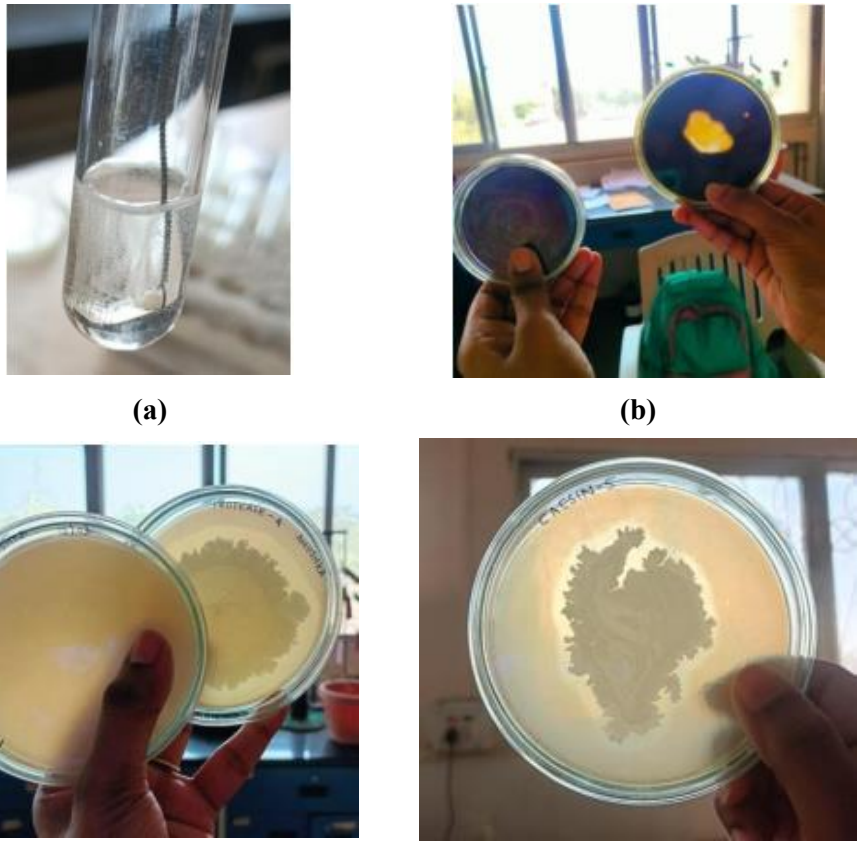


Figure 2: Sugar test (a) acid & gas production positive, (b) acid & gas production negative

Enzyme profiling (Table 4; Figures 3,4) showed production of catalase, amylase, protease, and caseinase by all but irregular gelatinase production by the isolates. Nutrient cycling is a significant feature of enzyme secretion and has been reported for PSB such as Pseudomonas cepacia and Enterobacter agglomerans from salt environments [19].

Table 4: Enzyme profile

	APP1	APP2	APP3	APP4	APP5	APP6	APP7	APP8
Catalase	+	+	+	+	+	+	+	+
Amylase	+	+	+	+	+	+	+	+
Gelatinase	-	-	-	+	+	-	+	-
Protease	+	+	+	+	+	+	+	+
Caesin	+	+	+	+	+	+	+	+



(a) **(b)**

(c) **(d)**

Figure 3: Enzyme profiling tests (a) catalase test positive, (b) amylase test positive, (c) protease test positive, (d) casein test positive

(a) (b)

Figure 4: Gelatin liquification test: (a) negative, (b) positive

Biochemical tests (Table 5) confirmed metabolic diversification. Phytase production by every isolate proved phosphorus mineralization potential under salt conditions [20]. Negative MR, VP, and nitrate reduction tests confirm non-fermentative or selective metabolic patterns, congruent with other PGPR halotolerant reports [21].

Table 5: Biochemical tests

	APP1	APP2	APP3	APP4	APP5	APP6	APP7	APP8
MR Test	-	-	-	-	-	-	-	-
VP Test	-	-	-	-	-	-	-	-
Citrate Test	-	-	-	+	-	+	+	-
Nitrate Test	-	-	-	-	-	-	-	-
Phytase Test	+	+	+	+	+	+	+	+

Identification of Species

ABIS Online and phenotypic tests showed the isolates to be primarily *Pseudomonas cepacia, Enterobacter agglomerans, Paenibacillus validus, Chryseomonas luteola*, and *Serratia ficaria* (Section 4.3). Associated genera have been isolated from salt soil and reported as efficient PSB with different plant growth-promoting attributes [22].

Effect of PSB on Phosphate Solubilization Under Saline Conditions

The screened isolates created well-defined halos at salinity, exhibiting efficient phosphate solubilization. The reason for this was the yield of organic acids such as gluconic and citric acids that possess the potential of chelating cations and liberating phosphate [23]. Similar mechanisms have been achieved in halotolerant Pseudomonas and Enterobacter strains [24].

High salinity may slow down the growth of bacteria and enzyme function, but halotolerant PSB tend to adjust by producing exopolysaccharide (EPS) and accumulating compatible solutes [25], as investigated in this paper.

Biofertilizer Production and Effectiveness

Preparation of the biofertilizer (Figures 5) from the isolate APP5 (*Enterobacter agglomerans*) greatly enhanced germination and seedling increase of mung bean, moth bean, and pigeon pea under salt conditions (Tables 6 & 7; Figure 6). Enhancements of germination by 62–145% in length of shoot are comparable to previous reports of biofertilizers using halotolerant plant growth-promoting bacteria (PSB) [26].

Figure 5: Production broth of PSB

Table 6: Seed germination assay

Seed Type	Control Root	Control Shoot	Test Root	Test Shoot
After 48 hours				
Mung (*Vigna radiata*)	1.4 cm	3.5 cm	3 cm	5 cm
Moth beans (*Vigna aconitifolia*)	2.3 cm	5 cm	7.3 cm	5.5 cm
Pigeon pea (*Cajanus cajan*)	1 cm	1.5 cm	1.4 cm	1.5 cm
Wheat (*Triticum*)	0.9 cm	0.4 cm	0.2 cm	0.3 cm
After 72 hours				
Mung (*Vigna radiata*)	1.5 cm	6.8 cm	5.8 cm	11 cm
Moth beans (*Vigna aconitifolia*)	2.9 cm	6.6 cm	5.4 cm	11.3 cm
Pigeon pea (*Cajanus cajan*)	1.5 cm	2 cm	2.9 cm	4.9 cm
Wheat (*Triticum*)	2.5 cm	2 cm	1 cm	0.4 cm

Table 7: % increment in root and shoot length of seed germination

	% Increment in Root Length	% Increment in Shoot Length
Mung (*Vigna radiata*)	283	62
Moth beans (*Vigna aconitifolia*)	86	71
Pigeon pea (*Cajanus cajan*)	93	145

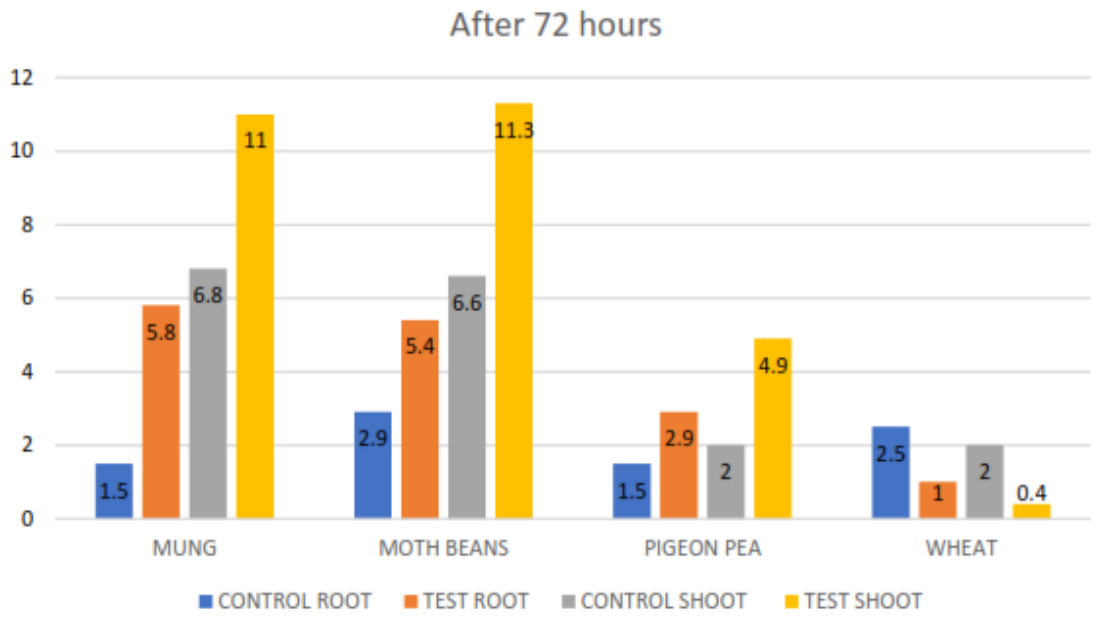


Figure 6: Effect of prepared bioinoculum on shoot length, root length

These effects have been a result of increase in phosphorus availability, synthesis of IAA and siderophore, and elongation of roots, as indicated by Sridevi *et al.* (2012) and Rashid *et al.* (2004). The results justify the potential of halotolerant PSB as a means of providing biofertilizers for sustainable yields in salinized soils.

Conclusion:

The germination test by using a biofertilizer made of halotolerant phosphate-solubilizing bacteria (PSB) recorded a substantial positive impact on germination and initial seedling development of mung bean (*Vigna radiata*), moth bean (*Vigna aconitifolia*), and pigeon pea. Comparable increases in germination percentage and seedling vigour by PSB treatment have been documented for various other leguminous species subjected to salinity stress [27].

The increase likely results from improved (*Cajanus cajan*). Phosphorus availability, organic acid production, and other plant growth-promoting activities like IAA and siderophore synthesis, extensively reported for halotolerant *Pseudomonas* and Enterobacter strains [28]. The results justify the promise of halotolerant PSB-based biofertilizers for enhancing crop establishment, more so in salinized or nutrient-deficient fields, toward the sustainable agriculture objectives of Vassilev & Vassileva (2003).

Indeed, the biofertilizer did not function on wheat (*Triticum aestivum*) and indicated an adverse impact on germination, and this may have been because of microbial crop-specific responses or salt sensitivity [29]. Seeds of peanut (*Arachis hypogaea*), chickpea (*Cicer arietinum*), brown chickpea (Cicer), and green pea (*Pisum sativum*) failed to germinate in either treatment, and it is likely that either salinity levels or elements of dormancy for the seeds functioned as an inhibitor [30].

Scale-field evaluation and crop-specific optimization of formulation are suggested for the confirmation of these results and widening the scope of application of halotolerant PSB biofertilizers under various agro-ecological conditions.

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Conflict of Interest:

The authors declare no conflict of interest.

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