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Stress Tolerant Phosphate Solubilizing Bacteria From The Rhizospheric Soils of Himachal Pradesh

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Abstract

Plant growth promoting rhizobacteria (PGPR) are one of the important sustainable tool for healthy agriculture which not only increase crop production but also improve the biological health of soil. Rhizospheric soil has high and diverse PGPR diversity which faces inevitable stress conditions. So, to perform excellently in the field conditions, the PGPR isolates must be resistant to different types of environmental stresses. Keeping this in view, in the present study a total of 33 bacterial strains were isolated from the rhizospheric soil of *Triticum aestivum* collected from different locations of Himachal Pradesh (India). Based on qualitative phospahte (P) solubilization assay the isolate PSB R1 (19mm) showed highest solubilization followed by PSB-C1 (17mm). By plate assay, on the tenth day of incubation, isolate PSB-C3 (242.86%) showed highest percent solubilization efficiency and isolate PSB-R9 showed 216.66% solubilization efficiency. Based on quantitative P-solubilization assay, highest P-solubilization was observed in case of PSB- R13 (470.5µg/ml) followed by PSB- P7 (425.0µg/ml). Whereas, isolate PSB-R9 showed 389.5µg/ml P-solubilization. The screened isolates were further subjected to various plant growth promoting tests. Maximum IAA production was showed by PSB- R9 (121µg/ml) followed by PSB-P2 (117µg/ml). The isolate PSB- R9 also showed highest production of siderophores (71.29%) as well as showed HCN production and strong production of ammonia. The isolate PSB-R9 showed antifungal activity against *Fusarium spp, Rizhoctinia spp.* and *Sclerotium spp*. Based on biochemical identification tests, the best efficient isolate PSB-R9 was found to be *Micrococcus spp*. The isolate PSB-R9 was further subjected to various stress conditions like P-solubilization in the presence of different conc. of NaCl (2%, 5% and 10%), pH (4.5, 5.5 and 8.5), temperature (4°C 25°C, and 37°C). The results showed that isolate PSB-R9 solubilized phosphorus efficiently under various stress conditions. So, overall isolate PSB-R9 was found to be most efficient PGPR isolate which tolerate various stress conditions. Therefore, isolate PSB-R9 could a potential candidate for the bioinculant formulation which can work efficiently in various agroclimatic zones.

Keywords: PGPR, Rhizosphere, Phospahte solubilization, Indole acetic acid

INTRODUCTION

Soil is an important component of terrestrial ecosystem. Soil is enriched with various microscopic biological forms like including fungi, algae, bacteria, protozoa, actinomycetes. These microorganisms play an important role in decomposition process and therefore very important for nutrient recycling [1]. The plant root is in direct contact with the soil which helps in absorption of nutrients. The narrow zone of soil which sourrounds the root is known as rhizosphere. The rhizospheric microbial diversity is affected by nutrients (like amino acids,

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sugars, organic acids etc) present in root exudates [2,3,4,5,6]. In the rhizosphere diverse microbial population coexist of which bacterial population present in higher number [7]. These microbial population colonize in the rhizosphere and influence the plant growth [8.9].

For the plant growth, phosphorus (P) is one of the important macro nutrient [10]. Phosphorus play an important role in plant growth like increase in the production of seed and flower, progress in root formation, increases the strength of stem and stalk, improve the production quality and maturity of crop, increase the plant resistance against diseases and in legumes improves nitrogen fixation.

Although, large amount of P is available in the soil but due to fixation and less solubility, avery small part of P is available in the soil (1 ppm or 0.1%) which can be easily absorbed by plant roots [11.12]. To overcome this problem generally farmers used phosphatic fertilizers at high doses. But soon after application, a major part of the fertilizer immobilized rapidly which is not available to plants. In the acidic soils, iron and aluminium fix the phosphorus, on the other hand calcium is responsible for fixing phosphorus in alkaline soils [13].

Thus, plenty of Phosphorous is available in soil but in fixed form that can't be utilized by plants. Under such conditions Phosphate solubilizing microorganisms (PSM) play an important role in providing plant usable phosphorous by sustainable means. These microorganisms solubilize insoluble phosphorous by producing organic acids which dissolve phosphatic materials and also may produce chelating compounds which release phosphorus freely in the soil [14,15]. The insoluble P is solubilized by diverse type of microbes like fungi, AM fungi, actinomycetes, and bacteria [16,17]. In the soil on the whole 1 to 10 percent population of bacteria and 0.1 to 0.5 percent of fungi constitute phosphate solubilizing population [18]. These PSB generally belong to the genera *Pseudomonas, Enterobacter, Bacillus, Penicillium and Aspergillus* that can solubilize insoluble phosphorous [19].

The PSM not only provides P but also improve the plant growth by producing various plant growth promoting compounds like siderophores, indole acetic acid, gibberlline, Cytokines etc [20,21,22], and kills soil borne pathogens and thus protect the plants [23,24]. Therefore, those bacteria which are favourable for plant growths and living in the rhizosphere are known as plant growth promoting rhizobacteria (PGPR). There are many environmental conditions which influence the growth of these PGPR such as salts, temperature and pH, heavy metals etc. All these types of stresses which are present in the environment have great impact on efficiency of PGPR [25]. Generally, these types of stresses either kill the microbes or forcing them to enter into dormancy state [26]. So, the indigenous PSM which have the ability to tolerate these stressful conditions and shows PGPR activity will play an important role as bioinoculant under stress conditions of various agroclimatics zones. Therefore, present study was designed to isolate and screen stress tolerant PSB from the rhizospheric soil.

MATERIAL AND METHODS

Collection of soil samples

Soil samples were carefully collected from the rhizosphere of *Triticum aestivum* (Wheat) plants collected from Palampur, Chamba, Rohru and Solan areas of Himachal Pradesh (India). The rhizospheric soil samples were collected in sterilized polybags by carefully uprooting of the plant and processed for the isolation of phosphate solubilizing bacteria (PSB).

Isolation of P-solubilizing bacteria

Serial dilution technique was used to isolate phosphate solubilizing bacteria from the soil samples and appropriate dilutions were spread over Pikovskaya's agar medium and plates were

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incubated at 30°C±1°C for 24-48 hrs. Colonies showing halo zone were considered as P-solubilizer and colonies were purified by restreaking and maintained on nutrient agar slants at 4°C for further microbiological analysis.

Screening of P-solubilizing bacteria

Qualitative plate assay screening

The isolated strains were spot inoculated on Pikovskaya's agar plates. Plates were incubated at 30°C ±1°C for 10 days and zone of solubilization was measured after every 24 hrs up to 10 days. The solubilization efficiency (E) of these isolates [27] was calculated at 10th day of incubation by using following formula:

$$\text{Solubilization efficiency (\%)} = \frac{Z-C}{C} \times 100$$

where,

Z= Zone of solubilization

C=Diameter of colony

The isolates that show 5mm or greater than 5mm of zone of solubilization was further subjected to quantitative assay of P-solubilization.

Quantitative P-solubilization assay

Jackson [28] and Nautiyal [29] methodology was adapted for the quantification of inorganic phosphate solubilization. Bacterial isolates were inoculated in 100 ml sterile National Botanical Research Institute's Phosphate (NBRIP) broth containing 0.5% tricalcium phosphate (TCP) in 250 ml Erlenmeyer flask and incubated at 30°C±1°C at 180 rpm in shaker incubator for 12 days. Under similar conditions the uninoculated control was also keept. After regular interval of time, small aliquots of culture sample was taken out and observed for P-solubilization and pH change. The yellow colour developed was read at 430 nm in spectrophotometer. The P- solubilized by the isolate was calculated from standard curve prepared by using potassium dihydrogen orthophosphate (KH_2PO_4).

PGPR activity

Production and estimation of Indole acetic acid (Patten and Glick, [30])

The modifyied Luria Bertani broth with different concentration of L- tryptophan (0, 50, 100, 200 µg/ml) was inoculated with screened isolates and incubated at 30°C±1°C for 72 hrs. Centrifugation at 8000 rpm for 25 min at 4°C was done to harvest the cells. Salkowski's reagent (4ml) and one to two drops of H_3PO_4 was added to the supernatant (1 ml). Formation of pink color indicates indole acid production and absorbance was taken at 530 nm in spectrophotometer. Quantity of IAA (Indole acetic acid) was determined from the standard IAA curve.

Detection of microbial siderophore production

i. Qualitative test (plate assay)

For the detection of siderophore, chrome azurol sulfonate (CAS) assay was performed as described earlier by Schwyn and Neilands, [31]. The plates were spot inoculated with bacterial cultures and incubated at 30°C±1°C for 24-48 hrs. The cultures showed yellow orange haloes around the growth was considered as siderophore producers.

ii. Quantitative spectrophotometric assay

The assay given by Payne, [32] was used to quantify the siderophore by CAS-shuttle assay. The absorbance of color obtained was determined using the spectrophotometer at 630 nm. The

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following formula is used to quantify the siderophore production: [(Ar -As)/Ar] x 100, where Ar is the absorbance at 630 nm of reference and As is the absorbance at 630 nm of the sample.

HCN production (Lata, [33]; Baker and Schippers, [34])

Nutrient agar medium amended with glycine (4.4gm/lit) was used to streaked the screened bacterial isolates. In the lid of plate whattman filter paper disc soaked in 0.5% picric acid (0.5% picric acid in 2% sodium carbonate) was placed and parafilm was used to seal the plates. Sealed plates were incubated for 4 days at 30°C±1°C. Color of paper disk changes from deep yellow to dark brown indicates HCN production.

Ammonia production

The protocol of Cappuccino and Sherman [35] was used to detect the ammonia production by all the screened bacterial isolates. Ten ml peptone broth was inoculated with log phase bacterial culture and incubated in shaking incubator at 30°C±1°C for 48 h. After incubation 0.5 ml of Nessler's reagent was added. The development of faint yellow to dark brown color indicates the production of ammonia.

Antifungal activity

P-solubilizing isolates were checked for their antifungal activity by Agar streak method. Test isolates were streaked below the centre of a prepared nutrient agar plate and fungal mycelium was inoculated in centre of the same plate. The plates were incubated at 30°C±1°C for 3-4 days. Plates were checked for inhibition of fungal growth at regular intervals of time for various plant pathogenic fungi.

Biochemical identification

The most efficient P-solubilizer was indentified on the basis of its biochemical and morphological characteristic's as mentioned in the Bergey's Manual of Systematic Bacteriology [36].

Effect of stress conditions on phosphate solubilizing activity

Phosphate solubilization was determined under different stress conditions like NaCl (2%, 5% and 10%), pH (4.5, 5.5 and 8.5), and temperature (4°C, 25°C and 37°C). NBRIP broth with different salt and pH conditions was inoculated with most efficient bacterial strain and incubated at 30°C±1°C for 12 days. Whereas, for temperature stress conditions, flasks containing NBRIP broth was inoculated with most efficient strain and incubated at respective stress temperatures. The phosphate solubilization was quantified as described in section "Screening of P-solubilizing bacteria".

RESULTS AND DISSCUSION

Isolation of phosphate solubilizing bacteria from *Triticum aestivum* (wheat) rhizosphere

Root exudates directly affect the rhizospheric soil which is in direct contact with surface of root. Plant roots exuded generally contain organic contents such as carbohydrates, flavonoids, organic acids, nucleotides, vitamins, enzymes, volatile compounds, and hormones in the rhizosphere and subsequently increased the microbial activity [37]. Because of this reason, in the present study rhizospheric soil samples of *Triticum aestivum* plant was used to isolate phosphate (P)-solubilizing bacteria (**Fig. 1**).

A total of thirty three bacterial strains were isolated from rhizospheric soils of *Triticum aestivum* plant. Out of which 8, 3, 8 and 14 bacterial strains were isolated from Solan, Chamba, Palampur and Rohru respectively (**Table1**). Earlier workers also isolated P- solubilizer from the rhizosphereic soil of various crop [38,39,40].

Screening of P-solubilizing bacteria

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Evaluation of P-solubilzing acitivity of bacterial strains using qualitative method

Rhizosphere is generally reported to have high number of phosphate-solubilizing bacteria [41]. *Bacillus* and *Pseudomonas* are most efficient and common reported P-solubilizers. The isolated 33 P- solubilizers were qualitatively screened for P-solubilization activity (**Fig. 2**). The highest zone of solubilization was observed (**Table 2**) in isolate PSB R1 (19mm) followed by PSB-C1 (17mm), PSB-C2 (17mm), PSB-C3 (17mm) and PSB-R9 (13), The minimum zone of solubilization was showed by two isolates PSB-S7 (2mm) and PSB-R14 (2mm) on Pikovskaya's (PVK) agar. Solubilization efficieny of all the isolated P- solubilizing strains were calculated at 10^{th} day of incubation (**Table 3**). Highest P- solubilization efficieny was observed in case of PSB-C3 (242.86%) and PSB-P1 (242.86%) followed by PSB-R1 (237.5%) and PSB-R9 (216.66%). Least solubilization efficieny was observed in case of isolate PSB- S7 (14.26%). Out of 33 isolates only 21 strains which showed more than 5mm zone of solubilization were further studied.

Table 1. Location of sample collection and designation of isolated phosphate (P) - solubilizer

S.No	Location	No. of P-solubilizer isolated	Designation
1.	Solan	8	PSB-S1, PSB-S2, PSB-S3, PSB-S4, PSB-S5, PSB-S6, PSB-S7, PSB-S8
2.	Chamba	3	PSB-C1, PSB-C2, PSB-C3
3.	Palampur	8	PSB-P1, PSB-P2, PSB-P3, PSB-P4, PSB-P5, PSB-P6, PSB-P7, PSB-P8
4.	Rohru	14	PSB-R1, PSB-R2, PSB-R3, PSB-R4, RSB-R5, PSB-R6, PSB-R7, PSB-R7, PSB-R8, PSB-R9, PSB-R10, PSB-R11, PSB-R12, PSB-R13, PSB-R14

Table 2. Phosphate- solubilization (mm) by various strains at different days of incubation

STRAINS	DAYS OF INCUBATIONS									
	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th
PSB- S1	3	3	4	4	5	5	5	6	6	6
PSB- S2	2	6	3	6	7	7	7	7	7	7
PSB- S3	-	-	-	-	-	-	-	-	-	-
PSB- S4	3	3	3	3	4	4	6	7	7	7
PSB- S5	2	3	2	2	2	3	3	3	3	3
PSB- S6	3	3	1	-	-	-	-	-	-	-
PSB- S7	-	3	1	2	3	3	3	2	2	2

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PSB- S8	**-**	**2**	**1**	**2**	**2**	**2**	**2**	**2**	**3**	**3**
PSB- C1	**3**	**5**	**9**	**11**	**12**	**13**	**15**	**17**	**17**	**17**
PSB- C2	**3**	**4**	**7**	**9**	**12**	**12**	**15**	**16**	**17**	**17**
PSB- C3	**3**	**5**	**9**	**10**	**12**	**12**	**14**	**15**	**16**	**17**
PSB- P1	**3**	**3**	**7**	**8**	**10**	**12**	**13**	**14**	**16**	**17**
PSB- P2	**2**	**2**	**3**	**4**	**5**	**5**	**6**	**7**	**7**	**8**
PSB- P3	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
PSB- P4	**-**	**-**	**-**	**2**	**3**	**3**	**4**	**9**	**9**	**11**
PSB- P5	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
PSB- P6	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
PSB- P7	**2**	**5**	**6**	**7**	**9**	**10**	**11**	**13**	**14**	**14**
PSB- P8	**1**	**2**	**4**	**5**	**7**	**9**	**10**	**10**	**13**	**14**
PSB- R1	**6**	**6**	**12**	**14**	**14**	**16**	**17**	**18**	**19**	**19**
PSB- R2	**4**	**7**	**6**	**7**	**8**	**8**	**8**	**8**	**9**	**9**
PSB- R3	**4**	**9**	**9**	**12**	**13**	**10**	**10**	**10**	**11**	**11**
PSB- R4	**-**	**-**	**2**	**4**	**4**	**4**	**5**	**5**	**5**	**6**
PSB- R5	**-**	**-**	**2**	**4**	**4**	**4**	**4**	**4**	**5**	**5**
PSB- R6	**2**	**2**	**4**	**4**	**5**	**5**	**7**	**7**	**7**	**6**
PSB- R7	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
PSB- R8	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
PSB- R9	**1**	**2**	**4**	**5**	**6**	**8**	**10**	**12**	**13**	**13**
PSB-R10	**1**	**1**	**2**	**2**	**2**	**5**	**6**	**5**	**5**	**5**
PSB-R11	**1**	**1**	**2**	**2**	**3**	**5**	**5**	**5**	**7**	**8**
PSB-R12	**-**	**2**	**2**	**1**	**1**	**3**	**2**	**2**	**3**	**4**
PSB-R13	**-**	**1**	**4**	**4**	**4**	**5**	**5**	**6**	**9**	**10**
PSB-R14	**-**	**3**	**3**	**1**	**1**	**1**	**2**	**2**	**-**	**2**

Table 3. Phosphate solubilizing efficiency of PSB strains at 10^{th} day of incubation

S.NO	STRAINS	TOTAL ZONE (mm)	COLONY SIZE (mm)	HALO ZONE (mm)	SOLUBILIZATION EFFICIENCY (%)
1.	**PSB- S1**	**15**	**9**	**6**	**66.66%**
2.	**PSB- S2**	**18**	**11**	**7**	**63.64%**
3.	**PSB- S3**	**-**	**-**	**-**	**-**
4.	**PSB- S4**	**23**	**16**	**7**	**43.75%**
5.	**PSB- S5**	**15**	**12**	**3**	**25%**
6.	**PSB- S6**	**-**	**-**	**-**	**-**
7.	**PSB- S7**	**16**	**14**	**2**	**14.28%**
8.	**PSB- S8**	**15**	**12**	**3**	**25%**
9.	**PSB- C1**	**25**	**8**	**17**	**212.5%**
10.	**PSB- C2**	**26**	**9**	**17**	**188.89%**
11.	**PSB- C3**	**24**	**7**	**17**	**242.86%**
12.	**PSB- P1**	**24**	**7**	**17**	**242.86%**
13.	**PSB- P2**	**13**	**5**	**8**	**160%**

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14.	**PSB- P3**	**-**	**-**	**-**	**-**
15.	**PSB- P4**	**21**	**10**	**11**	**110%**
16.	**PSB- P5**	**-**	**-**	**-**	**-**
17.	**PSB- P6**	**-**	**-**	**-**	**-**
18.	**PSB- P7**	**21**	**7**	**14**	**200%**
19.	**PSB- P8**	**23**	**8**	**15**	**187.5%**
20.	**PSB- R1**	**27**	**8**	**19**	**237.5%**
21.	**PSB- R2**	**22**	**13**	**9**	**69.23%**
22.	**PSB- R3**	**27**	**16**	**11**	**68.75%**
23.	**PSB- R4**	**15**	**9**	**6**	**75%**
24.	**PSB- R5**	**13**	**8**	**5**	**62.5%**
25.	**PSB- R6**	**14**	**8**	**6**	**75%**
26.	**PSB- R7**	**-**	**-**	**-**	**-**
27.	**PSB- R8**	**-**	**-**	**-**	**-**
28.	**PSB- R9**	**19**	**6**	13	**216.66%**
29.	**PSB- R10**	**17**	**12**	**5**	**41.66%**
30.	**PSB- R11**	**21**	**13**	**8**	**61.53%**
31.	**PSB- R12**	**21**	**17**	**4**	**23.52%**
32.	**PSB-R13**	**26**	**16**	**10**	**62.5%**
33.	**PSB-R14**	**30**	**28**	**2**	**25%**

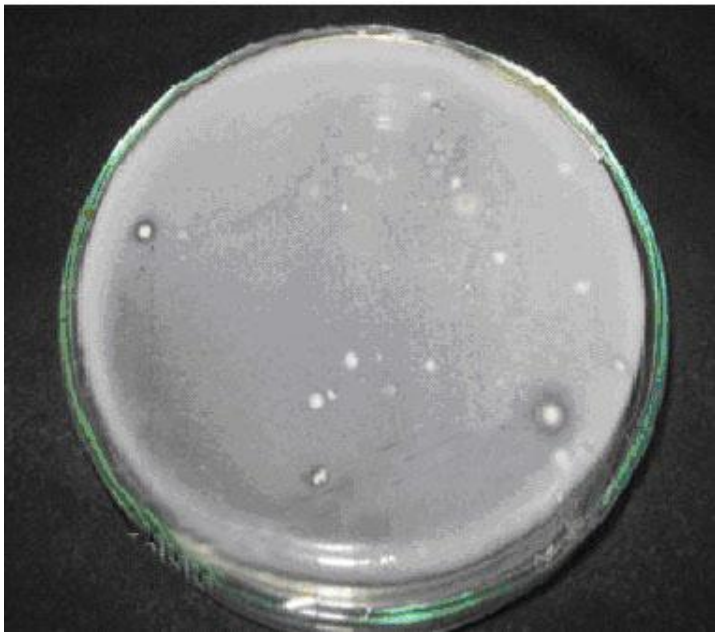

Fig. 1. Colonies of Phosphate Solubilizing Bacteria on Pikovskaya's Agar plate.

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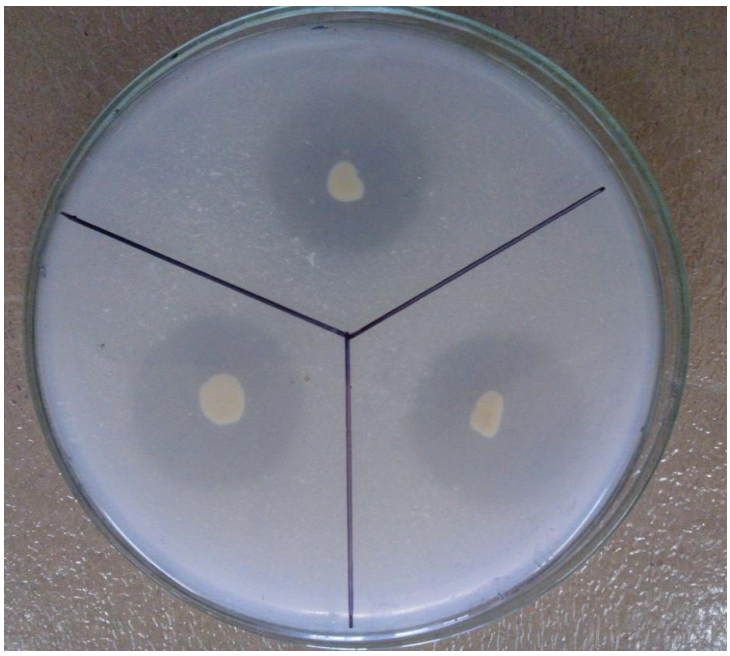

Fig. 2. Qualitative plate assay of phosphate- solubilization by the isolated bacteria on Pikovskaya's Agar plate.

Evaluation of P-solubilzing acitivity of bacterial strains using Quantitative method

In the literature already it is mentioned that many isolates on PVK media did not show halo zone around the colony but able to solubilize the insoluble phosphates in NBRIP broth [29]. Therefore, the isolates which show no halos or inconspicuous halos they fails in plate assay. The reason for this could be due to the organic acids secreted by the microbes diffuse into media at different rates depending upon species and type of acids secred [42].

Measurement of phosphate solubilization by plate assay is an indirect method which is not reliable. To achieve reliable results direct method of phosphate solubilization is done in NBRIP broth. Therefore, in the present study to get the reliable results the screened isolates were subjected to NBRIP broth assay for determing potential phosphate solubilizers.

The highest P-solubilization was observed in case of PSB- R13 (470.5µg/ml) followed by PSB-P7 (425.0µg/ml), PSB-R6 (410.5µg/ml), PSB-R9 (389.5µg/ml), PSB-R10 (380.5µg/ml), PSB-P4 (350.5µg/ml) and PSB-R11 (324.5µg/ml) at 9^{th} day of incubation. The least efficient isolate found was PSB-R4 which showed 65.0µg/ml of P-solubilization (**Table 4**). Isolated strains showed variations in the P-solubilization at different days of incubation. After reaching maximum P-solubilization, the quantity of P- solubilized decreased thereafter. This could be due to the microbial death in the medium [43]. Also, microbial cells consume the soluble phosphorus which leads to the reduction in soluble phosphorus after attaining the highest level [44].

The pH changes during the period of solubilization varies from 7.0- 3.04. In majority of cases the pH of medium lowered with the increase in the quantity of P-solubilization in culture broth and thereafter it increases. The production organic acids like citric, oxalic, succinic acid etc.leads to the decrease in pH [45]. The increase in pH on prolonged incubation is probably due to the death and lysis of microorganisms [43,46]. Some bacterial strains in the present study did not follow

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this trend of pH change. Here, fall in the pH was observed even when quantity of P-solubilization decreased after reaching maximum value. Similar observations were also noted by other workers [47,48,49,50].

Table 4. Quantitative measurement of P-solubilization acitivity and pH variation

S.No	STRAINS	P-SOLUBILIZATION (µg/ml)					pH				
		3rd day	7th day	9th day	11th day	13th day	3rd day	7th day	9th day	11th day	13th day
1.	PSB-S1	35.5	40.0	79.5	48.5	27.5	4.24	4.33	4.01	3.92	3.57
2.	PSB-S2	2.0	110.0	130.0	165.0	125.0	4.19	3.49	3.27	3.21	3.94
3.	PSB-S4	32.5	48.0	82.5	166.5	97.5	4.67	4.29	3.88	3.13	3.86
4.	PSB-C1	35.5	90.5	100.5	32.5	33.5	4.20	3.82	3.32	4.82	3.19
5.	PSB-C2	60.5	130.0	90.5	31.5	52.5	4.06	3.80	4.07	4.77	3.81
6.	PSB-C3	50.0	120.5	113.5	40.0	35.5	3.91	3.81	3.67	4.79	3.72
7.	PSB-P1	7.0	45.5	105.0	75.0	125.0	5.17	4.41	3.83	3.75	3.41
8.	PSB-P2	8.0	65.5	320.5	295.5	137.5	4.62	3.64	3.34	3.76	3.74
9.	PSB-P4	5.0	62.5	350.5	196.5	131.5	4.79	3.75	3.75	3.81	4.08
10.	PSB-P7	1.0	85.0	425.0	220.5	68.5	4.46	3.56	3.59	3.77	3.55
11.	PSB-P8	9.0	33.5	261.5	175.5	66.5	4.44	3.81	3.84	3.70	3.79
12.	PSB-R1	89.5	110.0	198.5	129.5	203.5	3.61	3.51	3.24	3.20	3.04
13.	PSB-R2	5.0	28.0	173.5	15.5	15.0	4.61	4.95	3.84	3.14	4.61
14.	PSB-R3	60.5	67.5	75.5	31.0	68.5	4.55	4.94	4.01	3.92	3.57
15.	PSB-R4	31.5	55.0	65.0	36.0	53.5	4.64	4.62	3.93	3.44	3.77
16.	PSB-R5	15.5	45.5	270.0	170.0	100.5	4.26	3.49	3.66	3.91	3.75
17.	PSB-R6	1.0	37.5	410.5	163.5	80.0	3.60	3.68	3.87	3.78	3.55
18.	PSB-R9	1.0	120.5	389.5	213.5	77.5	4.14	3.59	3.45	3.64	3.58
19.	PSB-R10	40.0	50.0	380.5	145.0	147.5	4.44	3.72	3.40	3.74	4.11

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20.	**PSB-R11**	**8.0**	**90.5**	**324.5**	**142.5**	**112.5**	**4.59**	**3.83**	**3.79**	**3.64**	**3.49**
21.	**PSB-R13**	**2.0**	**137.5**	**470.5**	**250.0**	**128.5**	**4.10**	**3.39**	**3.54**	**3.28**	**3.36**

PGPR Activities

Indole Acetic Acid (IAA) Production

IAA is a phytohormone which plays a crucial role as signaling molecule that regulate the development and growth of plant. Various conditions like growth stage of microbe, availability of substrate etc influences the production of IAA [51]. In plants and microbes, tryptophan acts as precursor for biosynthesis of IAA, as transamination and decarboxylation of tryptophan leads to production of IAA [52]. Therefore, in the present study tryptophan amended medium was used to evaluate the IAA production by the screened isolates. Maximum IAA production was showed by PSB- R9 (121µg/ml) followed by PSB-P2 (117µg/ml) and least IAA production showed by PSB-R11 (25µg/ml). While PSB- R4 do not produced IAA (**Table 5**). It is also observed that PSB-R13 strain which showed highest phosphate solubilization produced less amount of IAA *in vitro* as compared to PSB-R9. Samuel and Muthukkaruppan, [53] also studied the production of IAA by plant growth promoting fungi and rhizobacteria associated with mangrove, rice and effluent contaminated soil. It was observed that IAA production increases with increasing concentration of L- tryptophan. This could be due to L- tryptophan which acts as precursor of synthesis of IAA [54]. But level of IAA production varied from strain to strain. The production of IAA depends upon the strain of microorganisms used and their age [55]. Earlier workers [56,57,58] tested PSB for the production of Indole acetic acid associated with the rhizosphere.

Detection of microbial siderophore production

Generally, rhizopsheric soil is deficient in iron and under such conditions some microbes are able to produce siderophores which are iron-chelating agents [59]. These siderphores chelate the feric ions and make available for the microbial metabolism. The excess of unused feric ions are secreted by these microbes in the surrounding soils and make it available to plant roots [60]. The siderophore producing microbes showed not only efficient root colonization but also make available iron to the plants. Simultaneously, the chelating of iron in soil is delterious to phytopathogens as these pathogens require more iron for their growth [61].

Table 5. Production of IAA by isolates in presence of different L- tryptophan concentrations

S.No	STRAINS	0µg/ml	50µg/ml	100µg/ml	200µg/ml
1.	**PSB-S1**	**3**	**14**	**56**	**93**
2.	**PSB-S2**	**-**	**7**	**31**	**75.5**
3.	**PSB-S4**	**5**	**21**	**59**	**87**
4.	**PSB-C1**	**4**	**19**	**45**	**65.5**
5.	**PSB-C2**	**9**	**26**	**76.5**	**99**
6.	**PSB-C3**	**3**	**15**	**61.5**	**94**
7.	**PSB-P1**	**6**	**24**	**74.5**	**105**
8.	**PSB-P2**	**4**	**17**	**56**	**117**
9.	**PSB-P4**	**-**	**-**	**17**	**42.5**
10.	**PSB-P7**	**12**	**24**	**70**	**113**

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11.	**PSB-P8**	**-**	**-**	**20**	**32.5**
12.	**PSB-R1**	**-**	**-**	**-**	**37**
13.	**PSB-R2**	**-**	**-**	**-**	**23**
14.	**PSB-R3**	**7**	**34**	**78**	**107**
15.	**PSB-R4**	**-**	**-**	**-**	**-**
16.	**PSB-R5**	**2.5**	**12**	**41.5**	**89**
17.	**PSB-R6**	**4.5**	**23**	**56**	**111**
18.	**PSB-R9**	**10**	**27.5**	**89**	**121**
19.	**PSB-R10**	**5**	**13**	**43**	**96**
20.	**PSB-R11**	**-**	**-**	**-**	**25**
21.	**PSB-R13**	**-**	**11**	**45**	**101**

Table 6. Siderophore production by native Phosphate solubilizers

S.No	STRAINS	SIDEROPHORE PRODUCTION (Qualitative)	PERCENTAGE (%) (Quantitative)
1.	**PSB-S1**	**++**	**67.4%**
2.	**PSB-S2**	**+**	**17.49%**
3.	**PSB-S4**	**+**	**37.56%**
4.	**PSB-C1**	**+**	**28.4%**
5.	**PSB-C2**	**-**	**-**
6.	**PSB-C3**	**-**	**-**
7.	**PSB-P1**	**-**	**-**
8.	**PSB-P2**	**-**	**-**
9.	**PSB-P4**	**+**	**29.4%**
10.	**PSB-P7**	**-**	**-**
11.	**PSB-P8**	**-**	**-**
12.	**PSB-R1**	**_**	**-**
13.	**PSB-R2**	**++**	**53.88%**
14.	**PSB-R3**	**+**	**25.73%**
15.	**PSB-R4**	**+**	**36.94%**
16.	**PSB-R5**	**+**	**12.5%**
17.	**PSB-R6**	**-**	**-**
18.	**PSB-R9**	**+++**	**71.29%**
19.	**PSB-R10**	**+**	**34.5%**
20.	**PSB-R11**	**-**	**-**
21.	**PSB-R13**	**-**	**-**

- : No activity, ++ : Medium activity, +++: High activity

In the present study all the isolates were screened for qualitative and quantitative production of siderophore (**Table 6**). Maximum production was showed by PSB-R9 and the percentage of siderophore production in PSB-R9 was 71.29% (**Table 6**) followed by PSB-S1 (67.4%) and PSB-R2 (53.88%). On the other hand, PSB-R5 (12.5%) showed least production of siderophore. Also, it was observed that PSB-R13 did not produced siderophore. Other workers also detect the siderophore production by various phosphate solubilizing bacteria [62,63,64].

HCN production

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Some microorganisms are able to produce HCN which is reported to have biocontrol activity against various pathogens [65]. The source of carbon for HCN production is belived to be a glycine. Oxidation of glycine by two microbial enzymes, glycine:oxygen oxidoreductase and glycine dehydrogenase leads to the production of HCN and CO_2 [66].

In the present study all the isolates were screened for the production of HCN. Maximum activity was showed by isolates PSB-S2, PSB-R2 and PSB-R9 (**Table 7**) followed by isolates PSB-R4, PSB-P1 and PSB-C1 where as PSB-S4, PSB-C2, PSB-C3, PSB-P2, PSB-P4,PSB-P7, PSB-R1, PSB-R3, PSB-R6 and PSB-R11 do not produced HCN. It was observed from the **Table 7** that PSB-R13 showed less production of HCN as compared to the PSB-R9.

Other workers also detected HCN producing rhizospheric bacteria which help in plant growth and devlopment [67,68,69]. In one study psychrotolerant *Pseudomonas fragi* CS11RH1 showed hydrogen cyanide (HCN) production and use of this strain increased/improved the various plant growth parameters [70].

Ammonia production

There are various pathways of ammonia production in microbial cell and nitrogen fixation is one of the major pathways. Some plant growth promoting rhizobacteria have the ability to convert nitrogen into ammonia and excess of which secreted into the soil and responsible for plant growth [71].

Table 7. HCN and Ammonia production by native Phosphate- solubilizers

S.No	STRAINS	HCN PRODUCTION	AMMONIA PRODUCTION
1.	**PSB-S1**	+	+
2.	**PSB-S2**	+++	++
3.	**PSB-S4**	-	+++
4.	**PSB-C1**	++	++
5.	**PSB-C2**	-	++
6.	**PSB-C3**	-	+
7.	**PSB-P1**	++	+
8.	**PSB-P2**	-	++
9.	**PSB-P4**	-	+
10.	**PSB-P7**	-	++
11.	**PSB-P8**	+	++
12.	**PSB-R1**	-	+
13.	**PSB-R2**	+++	++
14.	**PSB-R3**	-	+++

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15.	**PSB-R4**	++	++
16.	**PSB-R5**	+	++
17.	**PSB-R6**	-	++
18.	**PSB-R9**	+++	+++
19.	**PSB-R10**	+	++
20.	**PSB-R11**	-	++
21.	**PSB-R13**	+	++

-: No activity, +: Less activity, ++: Medium activity, +++: High activity

Also, PGPR contain ACC deaminase which hydrolyze ACC, the immediate precursor of ethylene, to α-ketobutarate and ammonia, and thus confer the plant growth [72]. In the present study, all the 21 strains were screened for ammonia production (**Table 7**). The highest ammonia production was showed by isolates PSB-S4, PSB-R3 and PSB-R9. While, PSB-S1, PSB-C3, PSB-P1, PSB-P4 and PSB-R1 isolated strains showed less production of ammonia. On the other hand PSB-R13 showed medium production of ammonia *in vitro* as compared to the PSB-R9.

To overcome the problems of chemical fertilizers the application of PGPR is an alternative sustainable tool to preserve the biological properties of soil**.** Kumar *et al.,* [49]**,** Walpola and Yoon, [73] studied *in vitro* plant growth promoting activities of rhizobacteria isolated from rhizospheric soil of French bean plant.

Antifungal activity

Some PGPR have antifungal acitvity because they have the ability to secrete some chemicals which can kill or inhibit the growth of pathogen which indirectly increase the crop production [74]. PGPR also increase the immunity of plants by induced systemic resistance (ISR) by salicylic acid-dependent pathway. Mostly, *Bacillus* and *Pseudomonas* are reported to have antifungal activities and also they have the potential to induce ISR [75]. Mallesh, [76] studied rhizobacteria of *Ashwagandha* and *Coleus* and the mechanism carried by these bacteria to suppress the soil borne pathogens.

Therefore, in the present study antifungal activity of the screened isolates were tested against phytopathogenic fungi *Fusarium spp, Rizhoctinia spp.* and *Sclerotium spp* . It was observed that PSB-R9 showed high antifungal activity (**Table 8**) against the pathogenic fungi as compared to the PSB-R13 and other P-solubilizers. Other workers [77,78,79] also checked the antifungal activity of phosphate solubilizing bacteria against various plant pathogenic fungi.

Table 8. Antifungal activity of Phosphate solubilizers against plant pathogenic fungi

S.No	**STRAINS**	**PLANT PATHOGENIC FUNGI**		
		Fusarium spp.	***Rizhoctonia spp.***	***Sclerotium spp.***
1.	**PSB-S1**	+	-	-
2.	**PSB-S2**	++	+	+
3.	**PSB-S4**	-	-	-

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4.	**PSB-C1**	+	+	+
5.	**PSB-C2**	++	+	+
6.	**PSB-C3**	+	+	+
7.	**PSB-P1**	+	+	+
8.	**PSB-P2**	-	-	-
9.	**PSB-P4**	+	-	-
10.	**PSB-P7**	+	-	-
11.	**PSB-P8**	++	+	+
12.	**PSB-R1**	-	-	-
13.	**PSB-R2**	+	-	-
14.	**PSB-R3**	_	-	-
15.	**PSB-R4**	-	-	-
16.	**PSB-R5**	+	-	-
17.	**PSB-R6**	+	-	-
18.	**PSB-R9**	+++	+	+
19.	**PSB-R10**	+	-	-
20.	**PSB-R11**	-	-	-
21.	**PSB-R13**	+	-	+

- : No activity, +: Less Activity, ++: Medium Activity, +++: High Activity

Biochemical characterization of most efficient Phosphate solubilizer

Out of 33 isolates PSB-R9 was found to be most efficient strain. It was characterized on the basis of its biochemical and morphological characteristics. The native isolate was found to be gram +ve, non-motile cocci. It showed positive results for Oxidase, Catalase, Indole, Citrate, Urease and Nitrate reduction. While showed negative results for MR, VP and Gelatin liquefaction. It showed acid and gas production by fermenting Galactose and Lactose while in case of Cellobiose, Dextrose, Fructose, Maltose, Sucrose, Trehalose and xylose it only showed acid production. This isolate was not able to utilized Dulcitol, Inositol, Mannitol, Meribiose and Raffinose as a carbon source. Based on these results the isolate PSB-R9 was identified as *Micrococcus* spp.

Effect of stress conditions on phosphate solubilizing activity

Various abiotic stresses such as temperature, salinity, heavy metals, pesticides, soil pH, soil texture, soil nutrients and soil moisture are present in soil and microbes are very sensitive to these stresses [80]. Under stressful environments, organisms grow slowly which results in decreased solubilization of phosphorus. In present study the most efficient bacterial strain PSB-R9 were subjected to different stress conditions such as salt (NaCl), pH and temperature (**Table 9**).

PSB-R9 bacterial strain showed maximum phosphate solubilization in the presence of various salt concentrations. Maximum phosphate solubilization was observed at 2% NaCl (337.5µg/ml) followed by 5% NaCl (235.5 µg/ml) and 10% NaCl concentration (208.5 µg/ml). Generally, phosphate solubilization activity decreases as the concentration of sodium chloride increases

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because neutralization or chelation of proton may occur at high chloride ions concentraion or sometimes acid is produced [81].

Broth adjusted to different pH (4.5, 5.5 and 8.5) showed variation in phosphate solubilization with the maximum phosphate solubilization (228.5µg/ml) occurring at pH 5.5 (Table 9.) followed by pH 4.5 (228.5µg/ml) and pH 8.5 (203.5µg/ml). But there is decrease in phosphate solubilization with increase or decrease in pH because it affects bacterial growth by altering stability of proteins, rate of reaction, activity of enzyme, nucleic acid structure and other biological molecules [82]. PSB-R9 showed maximum phosphate solubilization at 25°C (285.5µg/ml) followed by 37°C (187.5µg/ml).

Although different temperature has been reported by earlier workers for solubilization, most of the studies showed 25°C as the optimum temperature for phosphate solubilization as compare to 37°C due to high production of metabolites by bacteria involved in phosphate solubilization [45,83]. This clearly suggests that bacteria adapted to local environment, environment temperature is directly linked to their metabolic activities [81].

Stress conditions decreases bacterial numbers colonizing root cells endophytically. So, rhizobacteria have to develop strategies to proliferate and survive under stressful conditions. To overcome these stresses, bacteria produces different types of osmoprotectants in their cytoplasm [84].

The activation of variousBacteria adapted to stress conditions by activation of stress defense pathways, which results in the production of LEA (Late embryogenesis abundant) proteins, chaperones, glutamate, proline and trehalose etc [85,86].

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Table 9. Phosphate solubilizing capacity of most efficient bacterial strain PSB-R9 at different stress conditions

Stress condition	P-solubilization (µg/ml)					pH				
	3rd day	7th day	9th day	11th day	13th day	3rd day	7th day	9th day	11th day	13th day
Salt										
2% NaCl	**65**	**115.5**	**203.5**	**92.5**	**337.5**	**5.43**	**5.23**	**4.28**	**4.08**	**4.39**
5% NaCl	**17.5**	**81.5**	**148.5**	**102.5**	**235.5**	**5.42**	**4.75**	**4.46**	**3.78**	**4.14**
10% NaCl	**66.5**	**70.5**	**130**	**71.5**	**208.5**	**5.46**	**4.74**	**4.06**	**4.11**	**4.29**
pH										
4.5	**94.5**	**137.5**	**113.5**	**67.5**	**228.5**	**5.37**	**4.12**	**4.01**	**3.29**	**3.50**
5.5	**351.5**	**319.5**	**285**	**285**	**251.5**	**4.55**	**2.73**	**3.00**	**4.18**	**3.16**
8.5	**123.5**	**203.5**	**137.5**	**121.5**	**127.5**	**5.16**	**3.88**	**3.79**	**3.87**	**3.56**
Temperature										
4°C	**-**	**-**	**-**	**-**	**-**	**5.60**	**5.56**	**5.49**	**5.32**	**5.29**
25°C	**78.5**	**86.5**	**52.5**	**83.5**	**285.5**	**5.38**	**5.11**	**4.36**	**4.27**	**4.07**
37°C	**36.5**	**111.5**	**40**	**50**	**187.5**	**5.68**	**5.11**	**4.45**	**4.39**	**4.20**

CONCLUSIONS

Phosphorus (P) is second most important nutrient for plant development and growth. Plenty of phosphorous is present in the rhizosphere but in insoluble or poorly soluble form. Under such conditions many chemical phosphatic fertilizers are incorporated in the field to augment the crop production but these chemical fertilizers directly affect the human health and microbial flora of the soil. To overcome this problem phosphate- solubilizing microorganisms (PSM) can be a

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sustainable tool to solubilize the insoluble P and make available to the plants. In the rhizosphere many abiotic and biotic stresses are presents and microorganisms are very sensitive to these stresses which also affect their metabolic efficiencies. So, to overcome this problem, these microorganisms synthesized various substances which help in them to tolerate these stress conditions and promote plant growth. Keeping all this in mind, the screened isolates were characterized for various plant growth promoting traits like IAA production, siderophore production, HCN production, ammonia production and antifungal activity.

In the present study a total thirty three phosphate solubilizing bacterial strains were isolated from rhizospheric soils of *Triticum aestivum* plant. Out of 33 isolates, PSB-R9 was found to be most efficient phosphate solubilizer (389.5µg/ml), which also produced IAA (121µg/ml) and siderophore (71.29%). This isolate also showed high production of HCN, Ammonia. PSB-R9 also showed antifungal activity against plant pathogenic fungi *Fusarium spp., Rhizhoctonia spp.* and *Sclerotium spp.* Under various abiotic stress conditions (salt, pH and temperature) this isolate showed phosphorous solubilization. So, PSB-R9 has a potential to be used for the development of bioinoculant which can be used under various agro climatic zone to increase crop yield.

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