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Assessing the potential of phosphate solubilizing bacteria isolated from rhizospheres of *Sorghum bicolor* grown in Surat, south Gujarat

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Abstract
Phosphate-solubilizing bacteria (PSB) convert insoluble soil phosphates into soluble phosphorus and are potential suppliers of soluble phosphorus to plants. The current study was undertaken to isolate, and screen PSBs from the rhizosphere of *Sorghum* (*s* L.) grown in Surat, Gujarat. A total of 21 bacterial strains were isolated. The isolated strains were screened for their phosphate solubilization efficacy using Pikovskaya's agar and broth. Six isolates were found to be phosphate solubilizers. The isolate SB14 showed maximum effectiveness in phosphate solubilization. Morphological and biochemical characterization identified the isolate as *Klebsiella* sp. Plant growth-promoting traits of the isolated SB14 strain were assessed by a pot culture experiment conducted with groundnut seeds (*Arachis hypogaea*) using seeds treated with the bioinoculant of the isolated strain; uninoculated seeds were used as controls. Significant enhancement in seed germination of the inoculated seeds was observed. A marked growth in the aerial parts was also recorded.

Keywords: *Sorghum bicolor* L, phosphate solubilizing bacteria, Pikovaskya's agar, *Arachis hypogaea*, bioinoculant

1. Introduction
Rhizosphere, the nutrient rich region of the soil surrounding the plant root harbours a varied range of microorganisms. Several studies have reported that bacteria are the most dominant inhabitants of the rhizosphere. These bacteria are known as rhizobacteria. The diverse range of root exudates attracts these bacteria and they in turn serve as a communicating molecule to initiate biological and physiological interactions between the soil microbiome and the plant roots [1, 2].

Phosphorus is one of the vital nutrients essential for plant growth and development. Most of the soil phosphorous is present in precipitated forms and cannot be absorbed by plants. Plants can only utilize phosphorus when it is converted to a soluble form. Phosphorus (P) deficiency is a major limitation in crop production. Some bacteria have mechanisms for the conversion of phosphorus into a soluble form. They are referred to as phosphate-solubilizing bacteria (PSB). PSBs enhance plant growth by making phosphorous available to the plants thereby promoting plant growth and development. Rhizobacteria are the most predominant microorganisms that solubilize mineral phosphate in nature, as compared to other microorganisms [3].

Sorghum bicolor (L.) is an important cereal and is the staple food of people belonging to numerous regions worldwide. It can withstand adverse climatic conditions and has the potential to yield under unexpected weather conditions. Previous studies has reported the rich bacterial community associated with *Sorghum bicolor* responsible for its enhanced growth. A large number of nitrogen-fixing bacteria belonging to several bacterial domains were reported to colonize the rhizosphere and boost the growth of the plant [4, 5, 6].

The current study aims to isolate and characterise PSB associated with the rhizospheric microbiome of *Sorghum bicolor* cultivated in the Surat region of South Gujarat.

2. Materials and methods
2.1 Collection of rhizospheric soil
The rhizospheric soil sample was collected from of *Sorghum bicolor* grown in Surat region of South Gujarat.

The root along with the soil was sealed in a UV-sterilized ziplock bag and carried to the laboratory for further study [7].

2.2 Isolation of the bacterial isolates
Bacterial strains were isolated from soil samples by the serial dilution technique. 0.1 mL of soil suspension was spread on Nutrient Agar Medium plates and incubated at 37 °C for 48 h. Fast-growing and differential colonies were selected for further studies. Pure single-colony isolates were obtained by repeated streaking of bacterial cultures on a fresh nutrient agar medium [8].

2.4 Screening of phosphate solubilizing bacteria
The rhizobacterial isolates were spot-inoculated in petri plates containing Pikovaskya's medium, which contains grams/litre of glucose (10.0 g), Ca_3 $(PO_4)_2$, 5.0 g, $(NH_4)_2SO_4$, 0.5 g, NaCl (0.2 g), and $MgSO_4$.7H2O (0.1 g), pH 7.3±2. The halo zone and colony diameters were measured after 7 days of incubation of the plates at 30ºC. Colonies of PSB were detected by the clear zones of solubilisation around them. The diameter of halo zones was used to calculate the solubilisation index (SI) using the following formula [9]:

Solubilization Index (SI) = (colony diameter + halo zone diameter) / colony diameter

2.4.2 Quantitative test
The determination of available phosphorous was performed using the phosphomolybdate blue colour method. The quantitative estimation of phosphate was determined in Pikovaskya's broth. The isolated colonies of PGPRs were inoculated in 30 ml of Pikovaskya's broth and kept in an orbital shaker at 37 °C for 7 days at 120 rpm. After incubation, the culture was harvested by centrifugation at 10,000 rpm for 10 minutes. After incubation, the medium was centrifuged at 10,000 rpm for 10 min. The supernatant was collected and the ascorbic acid reductant method the supernatant (One ml) was mixed with ammonium molybdate and a sulphonic acid solution. The absorbance measured at 570nm g UV-Visible spectrophotometer. The concentration of solubilized phosphate was calculated using standard curve of KH_2PO_4 [10, 11].

2.3 Morphological and biochemical characterization
Various colony characteristics such as colour, shape, elevation surface, margin, optical feature, and degree of growth were observed for characterization. For biochemical characterization of the isolates the methyl red test, the voges-Proskauer test, the citrate utilization test, the catalase test, the triple sugar iron agar test, and nitrate test [8].

2.5 Pot experiment
A pot assay was conducted using the most efficient phosphate solubilizing bacterial isolate on seed germination and growth pattern of groundnut (*Arachis hypogaea*) purchased from Bilimora Nursery and farm, Surat, Gujarat. The groundnut plant was authenticated by expert.

2.5.1 Preparation of Bacterial inoculum
The strain showing maximum phosphate solubilizing efficiency was grown in nutrient broth in a shaking incubator at 48±2 °C for 24 hours, with agitation at 180 rpm. The bacterial cell density in the suspension was adjusted to a final density of approximately 10^8 CFU ml^{-1} [12].

2.5.2 Inoculation of Groundnut seeds
Healthy groundnut seeds were surface sterilized with 0.1% $HgCl_2$ for two minutes followed by repeated rinsing with sterile distilled water to remove any excess sterilizing agent. Groundnut seeds were inoculated with the bacterial suspension for 30 minutes. The seeds inoculated with nutrient broth medium without bacterial suspension were used as control. Root length, shoot length fresh weight of root and shoot, number of nodules and number of roots of the germinated seeds were studied after 50 days [13].

3. Results
3.1 Isolation of the bacterial isolates
21 bacterial strains were isolated from rhizospheric soil of *Sorghum bicolor*. The strains were screened qualitatively for their ability to solubilize phosphorus and were further evaluated quantitatively.

Fig 1: Rhizospheric soil sample of *Sorghum bicolor* L.

3.2 *In vitro* screening of the phosphate solubilizing bacteria
3.2.1 Qualitative test
Six of the 21 bacterial isolates showed phosphate solubilization activity. The strains SB1, SB3, SB4, SB10, SB14 and SB17showed phosphate solubilization indexes as 4.5, 4.63, 4.25, 4.66, 3.83, 6.14, and 3.90 respectively. The highest phosphate solubilizing index was shown by the strain SB14.

Table 1: Screening of Phosphate solubilizing strains

Colony no	Colony diameter (Cm)	Zone diameter (cm)	Solubilization Index (SI)*
SB1	4.5 cm	16.0 cm	4.50 cm
SB3	2.2 cm	10.2 cm	4.63 cm
SB4	4.0 cm	13.0 cm	4.25 cm
SB10	3.0 cm	11.0 cm	4.66 cm
SB14	3.5 cm	18 cm	6.14 cm
SB17	5.0 cm	9.5 cm	3.90 cm

3.2.2 Quantitative test
Results of screening for clear zone formation showed SB14 and SB1, SB10 possessing maximum solubilizing index. The index for clear zone formation of these strains is shown in Table 1. SB14 strains gave the largest clear zone. Table.1. the solubilized phosphate concentration was 209.417±0.740 µg/ml. The phosphate solubilizing activities of all selected strains were determined quantitatively by the UV-VIS spectrophotometric method at 570 nm using KH_2PO_4 as the

standard (Table 2). SB 14 showed maximum solubilization ability. In the presence of sulphuric acid, phosphate solubilized by PSB SB 14 forms a yellow precipitate of ammonium phosphate molybdate after adding ammonium molybdate. This precipitate reacts with amino-naphthol-sulfonic acid, producing a molybdenum complex that turns the solution blue, indicating the presence of phosphorus. This method, known as the molybdenum blue method, is commonly employed for the detection and quantification of phosphorus in sample [11].

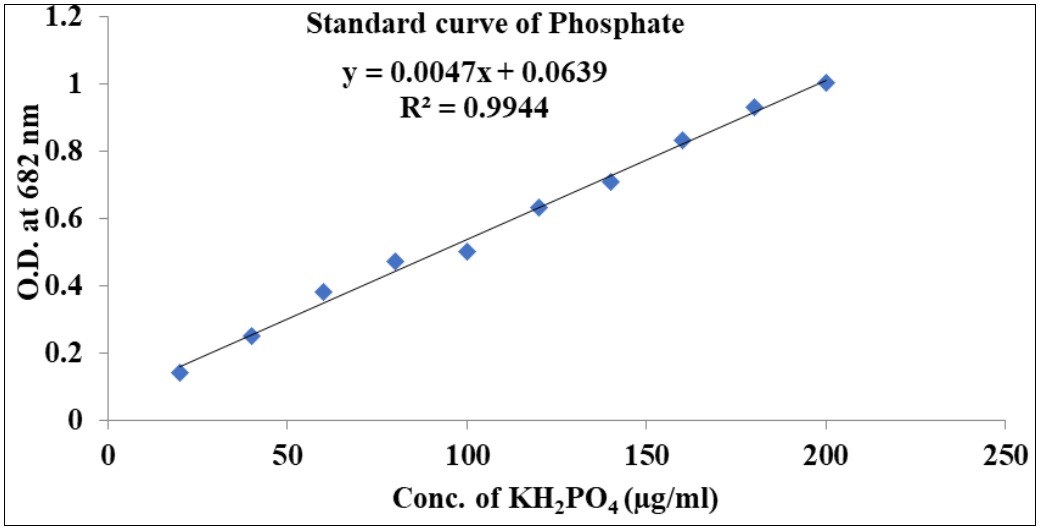

Fig 2: Standard Graph of Phosphate Concentrations

Table 2: Phosphate solubilizing activities of six most potent strains

S. No	Isolates of PSM	Soluble phosphate concentration (µg/ml)
1	SB1	209.25±0.14
2	SB3	123.75±0.25
3	SB4	159.166±0.083
4	SB10	206.5±0.144
5	SB14	209.417±0.740
6	SB17	147.833±0.682

*The result indicates mean of triplicate value± standard error

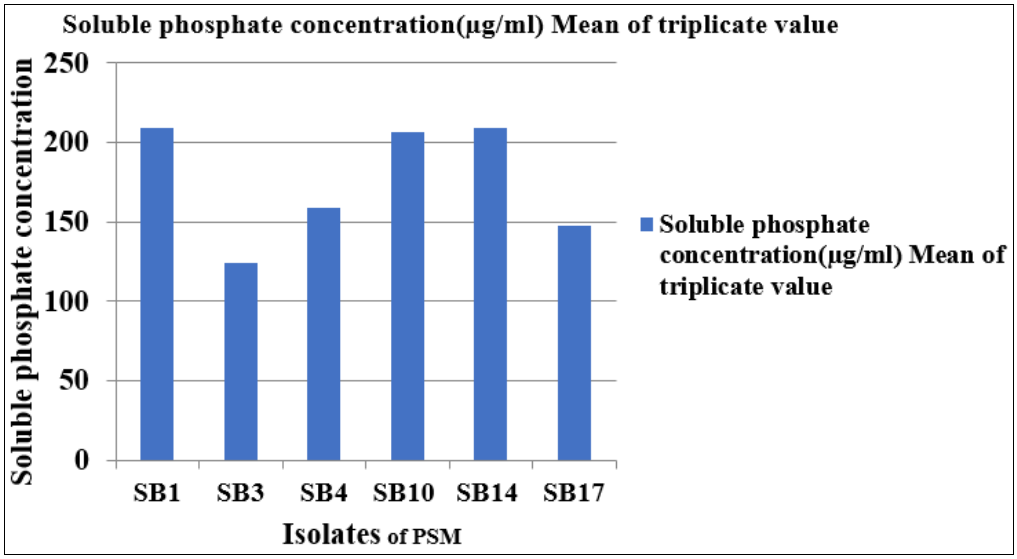

Fig 3: Phosphate solubilizing activities of the isolated strains

3.3 Morphological characterization

Phosphate solubilizing rhizospheric isolates was characterized by their cell shape and gram's reaction colony morphology for their tentative identification. The cell shape varies from the short rod to the cocci. The colony morphology varied from flat, effused, wavy, entire, erase or repand margin, moist, dry, butyrous, and dewdrop textures in nature, as shown in Table 3, and also varied in colony colours like peach, white, yellow, golden yellow, etc.

Table 3: Morphological characteristics of PSB isolates

Sr. No	Colony Size	Colony Shape	Colony margin	Colony elevation	Colony pigmentation	surface	constituency	Optical characteristic	Gram reaction
SB1	Small	Round	Entire	Convex	Red	Smooth	Moist	Translucent	-ve
SB3	Small	Round	Entire	Convex	White	Smooth	Moist	Translucent	-ve
SB4	Small	Round	Entire	Flat	Creamy white	Smooth	Moist	Translucent	+ve
SB10	Pinpoint	Round	Entire	Effused	White	Undulate	Moist	Vitreous	+ve
SB14	Pinpoint	Round	Entire	Flat	White	Punctuate	Moist	Porcelaneous	+ve
SB17	Small	Round	Entire	Raised	White	Smooth	Butyrous	Translucent	-ve

3.4 Biochemical Characterization

The biochemical test is an important method for the tentative identification of the isolates [9] All the PSB isolates were tested for biochemical for preliminary identification of rhizobacteria. Results for the biochemical test were shown in Table 4. Differential biochemical tests Methyl-red, vogus-proskauer, citrate utilization, nitrate reduction, gelatine hydrolysis, and TSI test results were observed after their respective incubation times. Two out of six isolates was found to have a positive result for the M-R test. One isolates gave a positive result for the V-P test. Three out of six isolates were found to have a positive result for the citrate utilization test. Three isolates gave positive results for the nitrate reduction test. Five isolates gave a positive result for TSI.

Table 4: Biochemical characteristics of the PSB isolates

	SB1	SB3	SB4	SB10	SB14	SB17
M-R	+	-	-	-	-	+
V-P	+	-	-	-	-	-
Indole	-	-	-	+	-	-
Nitrate	+	-	-	-	+	+
Gelatin	+	-	-	-	-	+
TSI	+	+	+	+	-	+
Citrate	+	-	+	-	-	+

+indicates presence, - *Indicates* absence

Based on the morphological characters and biochemical reaction, the phosphate solubilizing isolates were identified as SB1 *Serratia* sp., SB3 *Staphylococcus* sp, SB4 *Staphylococcus* sp, and SB10 *Bacillus* sp., and SB14 *Klebsiella* sp., SB17 *Proteus* sp.

SB14 *Klebsiella* sp. was observed to have maximum phosphate solubilizing capacity with psi of 6.14 cm and phosphate solubility of 209.417±0.740 µg/ml.

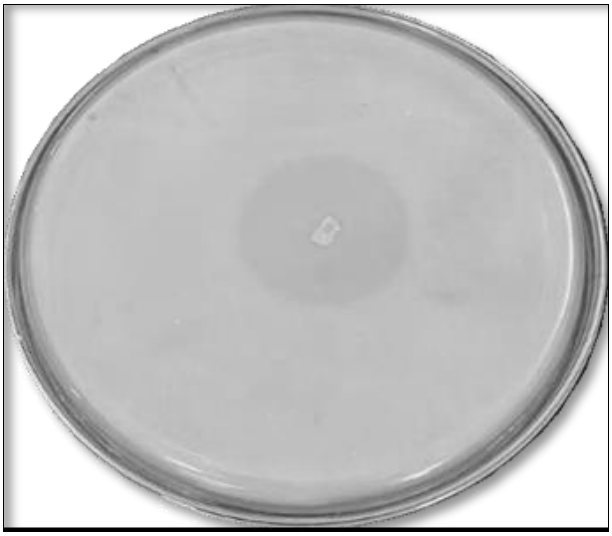

Fig 4: Qualitative analysis of phosphate solubilization of the strain SB 14 *Klebsiella* sp.

3.4 Pot Experiment

The comparisons between groundnut seeds treated with the SB14 strain and control plants reveal enhancement in the germination of the inoculated seeds. A substantial increase in root length (9.3 cm ±0.3) and shoot length (32.3 cm ±0.65) of the SB14 treatment plants was also observed after 50 days of inoculation. The mean root length and shoot length of the plants under control were recorded as of 7.43 cm ± 0.120 and 23.1 cm ± 0.519 respectively. SB14-treated plants showed increase in biomass (fresh weights) of roots (1.133 gm ± 0.012) and shoots (3.06 gm ± 0.378) compared to control at 0.233 gm ± 0.08 and 3.01 gm ± 0.060 respectively (Table 5).

Fig 3: Growth of control and SB14 treated groundnut after 50days

Table 5: Effect of SB14 on the ground nut plant

Sr.no	Length of root (cm)	Length of shoot (cm)	Fresh weight of root(gm)	Fresh weight of shoot(gm)	No of nodules	No. of roots
Control	7.433±0.120	23.1±0.519	0.233±0.08	3.01±0.060	3.333±1.201	37.666±0.881
SB14	9.3±0.3	32.3±0.650	1.133±0.012	3.06±0.378	10.666±0.881	77.666±0.666

*The result indicates mean of triplicate value± standard error

4. Discussions

The availability of phosphorous in soil is limited. There is constant need to improve soil P availability. Phosphate solubilizing bacteria (PSB) play an important role as microbial resources specialized in P cycling. rhizobacteria play an important role in phosphate solubilization. Many plant growth promoting rhizobacteria (PGPR) like *Pseudomonas, Azotobacter, Xanthomonas, Rhodococcus, Arthrobacter, Serratia, Chryseobacterium, Gordonia, Phyllobacterium, and Delftia* sp. have been categorized as PSB exhibiting substantial benefits for plant growth and yield [14].

In the current study, initial screening for the PSB isolates were based on the diameter of the halo zone formed. Further secondary screening of isolates based on quantitative parameters was conducted to identify the best phosphate solubilizing bacteria. Quantitative screening depended on their ability to solubilize phosphate from insoluble calcium phosphate, a phosphate source in Pikovskaya's medium. In our study, all bacterial isolates were screened for their phosphate solubilizing ability both qualitatively and quantitatively. In qualitative screening, a clear zone surrounding the bacterial isolates on Pikovskaya's agar medium indicated phosphate solubilization and is considered a positive test [15].

The isolates SB1, SB3, SB4, SB10, SB14, and SB17 gave positive results, as shown in Table 1. After qualitative estimation of phosphate solubilization, isolates were further evaluated quantitatively in Pikovskaya's broth medium. Six isolates showed P-solubilization on broth medium. A previous study with PSB strains from various zones of western Ethiopia using Pikovskaya's agar reported *Bacillus* sp and *Pseudomonas* sp. capable of maximum phosphate solubilization [16].

Four phosphate solubilizing bacteria identified by 16S rRNA sequencing as *Pseudomonas xantha* IAM 12356; *Burkholderia gladioli* R406; *Enterobacter hormaechei* EN 314; and *Serratia marcescens* A3, were isolated from the rhizosphere of *Aloe barbadensis* [17, 18].

The most efficient rhizospheric PSB identified in the present study using morphological and biochemical characteristics are SB1 *Serratia* sp., SB3 *Staphylococcus* sp, SB4 *Staphylococcus* sp, SB10 *Bacillus* sp., and SB14 *Klebsiella* sp., SB17 *Proteus* sp. are regarded as the major group of plant growth promoting rhizobacteria (PGPR) [19] The rhizobacteria belonging to the genera *Azotobacter, Acinetobacter, Klebsiella, Azatobacter, Azatospimillam,* etc., have been qualified as good PGPR [20, 21, 22]. Bacterial treatments are used to assess the plant growth-promoting traits of the rhizobacteria for the selection of the optimal bacterial strains [23]. In the present study pot experiment carried out with groundnut seeds inoculated with the most efficient PSB SB14 *Klebsiella* sp yielded good results as compared to the control set consisting of uninoculated seeds. A similar result of significant improvements in plant biomass compared to control plants of PGPR-treated groundnut plants. The study reported reductions in dry weight by 11% and fresh biomass by 19% in control plants compared to PGPR-treated plants. In a previous study, PGPR strains, specifically *Escherichia coli* DACG2, *Pseudomonas fluorescens DACG3*, and *Bukholderia sp.* DACG1 was found to produce a positive impact on plant growth in chickpea plants [24].

5. Conclusion

In the present study, six bacterial isolates showed phosphate solubilizing abilities. The strain SB14 *Klebsiella* sp. was found to be the most efficient. Using it as a bioinoculant in a pot experiment with the seeds of ground nut produced good results as compared to the control of untreated seeds. This strain possesses the functional potential of an environment-friendly alternative to toxic agrochemicals.

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