



Evaluation of *Bacillus subtilis* MRB4, as plant growth promoter and potential phosphate solubilizer under abiotic stress

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ABSTRACT

Exploitation of soil microbes to make fixed P and applied P available is years old practice to achieve high crop yield. Thirteen bacterial isolates capable of phosphate solubilization were isolated from the agricultural fields of Mahbubnagar, district of Telangana State, India, which were further tested for possessing plant growth promoting abilities and P solubilization potential under abiotic stress. Isolate MRB4, produced highest concentration of indole acetic acid i.e. 65 micro gram (μg)/ml, showed positive results for ammonia, hydrogen cyanide, cellulase, protease and exhibited promising antifungal activity. Solubilized tri calcium phosphate under various abiotic stress such as temperature 45°C (193 μg /ml), pH 9 (181 μg /ml), salt 8% (124 μg /ml), and Drought -0.49 matric potential (90 μg /ml). This potential isolate MRB4 is identified as *Bacillus subtilis* by 16S rRNA sequencing and deposited in the data bank with the accession number MK611797 and scanning electron microscope imaging is done.

1. INTRODUCTION

For biological growth and development, phosphorous (P) is the second most important macronutrient after nitrogen [1]. It shows significant role in seed development, ramification of root, and increases plant strength, thereby imparting disease resistance capacity and vitality to the plants [2]. Deficiency of P in the plants results in wilting of leaves, delayed maturity, reduced yield, and stunted growth [3]. 0.05% (w/w) content of P is present in average soil, and the P available to the plants is 0.1% of the total P reserve, low solubility, and fixation in soil makes it less available to plants [4]. To achieve maximum productivity, chemical fertilizers rich in soluble forms of P is made available to the soil. However, soluble P can be easily precipitated and get converted to forms having low solubility such as $\text{Ca}_3(\text{PO}_4)_2$, CaHPO_4 , AlPO_4 and FePO_4 , and thus becomes unavailable to plants [5,6]. To circumvent P deficiency due to fixation, excess P was applied by farmers [7], less to be utilized by plants and forcing major portion to be accumulated in soil causing

environmental and economic problem. [8,9], hence microbial application as biofertilizer is gaining momentum in agriculture [10], as availability of phosphates to plants and dynamics of P in soil is greatly influenced by soil microorganisms [11]. Dominant phosphate solubilizing bacteria (PSB) belongs to the Genera *Bacillus*, *Pseudomonas*, *Enterobacter*, *Serratia*, *Pantoea*, *Flavobacterium* etc. and dominant fungal genera are *Penicillium* and *Aspergillus* [12]. Majority of phosphate solubilizing microorganisms (PSM) solubilizes bound P complexes by extrusion of proton or by secretion of organic acids [13] which are most effective in releasing P from the soil [14].

In addition to P solubilization studies discussed above, soil microbes also supports growth of plant directly by N-fixation, ammonia production and by production of growth hormones for the plants. The indirect mechanism includes antibiotics production, extracellular production of enzymes which degrade cell wall of pathogens, hydrogen cyanide (HCN) and siderophore production, hence significantly promotes plant growth and improves plant health as evidenced by increase in vigor, seedling emergence and yield [15,16] Although PSMs are abundant in rhizosphere of most plants, but due to environmental factors such as pH, temperature, salinity and drought, survival and functionality of PSM is poor, hence making it necessary to isolate microbes from these

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conditions [15,17–19]. Therefore, the present research is designed to isolate and identify abiotic stress tolerant PSB from the arid regions of Telangana State, India and with other plant growth promoting (PGP) traits.

2. MATERIAL AND METHODS

2.1. Isolation of Rhizospheric PSB

The soil used for isolation of bacteria was collected from the agricultural fields of maize, rice, and ground nut in sterilized polythene bags, from the Mahbubnagar district of Telangana State, India. The serially diluted, soil samples were spread plated on nutrient agar and incubated at 30°C for 48 hours. Isolated, morphologically distinct colonies were selected and screened for their phosphate solubilization on National Botanical Research Institute Phosphate (NBRIP) agar medium [20] containing tri calcium phosphate (TCP) by point inoculation method. Plates were incubated at 30°C for 5 days. Colonies showing clear halo was selected and solubilization index was measured [21,22].

2.2. Quantitative Estimation of P Solubilized

Estimation of solubilized P was carried out by inoculating 1 ml of bacterial suspension (3×10^7 cells ml⁻¹) in 150 ml Erlenmeyer flask containing 50 ml NBRIP broth medium, flask were incubated for 7 days at 30°C. After incubation, broth was centrifuged at 10,000 rpm for 10 minutes [23]. Released free P in supernatant was estimated by Ames method [24].

2.3. Screening for Plant Growth Promoting Activities

2.3.1. Indole acetic acid (IAA) production

One milliliter of bacterial suspension was inoculated in 50 ml of Luria Bertani broth supplemented with L-Tryptophan [100 micro gram (µg)/ml], flasks were incubated at $28 \pm 2^\circ\text{C}$ for 48 hours, samples were centrifuged at 10,000 rpm for 10 minutes, IAA was estimated in the supernatant by the standard method of Gordon and Weber [23,25].

2.3.2. Ammonia production

Ten milliliter peptone broth was inoculated with 200 µl cultures suspension (3×10^7 cells ml⁻¹) of each PSB isolate and incubated at 30°C for 48 hours, 0.5 ml of Nessler's reagent was added to each tube. Deep orange to brown color development was indicative as a positive test for ammonia production [26].

2.3.3. Production of HCN

The method of Bakker and Schipper was employed for qualitative testing of PSB isolates for HCN production. Glycine supplemented, nutrient agar medium plates were prepared. Inoculated media plates were covered with Whatman No.1 Filter paper (saturated with picric acid and sodium carbonates) and incubated for 4 days, filter paper developed brown color considered positive for HCN production [27].

2.3.4. Protease

PSB isolates were inoculated on skim milk agar medium plates by point inoculation method. The development of clear zone, around the colonies indicated positive proteolytic activity [28].

2.3.5. Cellulase

PSB isolates were screened by inoculating over-night grown cultures on cellulose Congo-Red agar media. Discoloration of Congo-red around the colonies were considered as positive for cellulose degradation [29].

2.3.6. Antifungal activity

Potato dextrose agar was used to screened antifungal activity of PSB isolates against plant pathogens *Macrophomina phaseolina* and *Sclerotium rolfsii* by dual culture method [30].

2.4. Plant Growth Promotion Study by Paper Towel Experiment

Maize seeds (*Zea mays*) Aparanji-i variety, were surface sterilized as per the method described by Soni et al. [31]. The seeds were then left immersed in each suspension of PSB isolates (1×10^8 cells ml⁻¹) containing 1% carboxy-methylcellulose for 24 hours. Germination percentage was assessed as per International Seed Testing Association (ISTA, 1999) [32].

2.5. Identification

Microscopic identification was carried out by Gram staining, biochemical tests were performed as per the Bergey's manual of systematic Bacteriology [33].

2.6. P-Solubilization Under Abiotic Stress

Prominent PSB isolates were assessed for their tolerance to abiotic stress such as temperature (45°C), pH (9), salinity (8%) and drought (-0.49 Matric Potential). All experiments were done in triplicates using 50 ml NBRIP broth supplemented with TCP in 150 ml conical flask under various stressed conditions and flask were incubated for 7 days on orbital shaker at 150 rev¹ minutes. Volume of inoculum was adjusted to 3×10^{-7} ml⁻¹ [30].

2.7. Molecular Identification and Scanning Electron Microscope (SEM) Imaging

Molecular identification of potential PSB isolate was done at Eurofins Genomics Pvt. Ltd., Bengaluru and SEM image of the same was taken at Physics Department, Osmania University, Hyderabad, India.

2.8. Statistical Analysis

All experiments were carried out in triplicates ($n = 3$). Standard deviation was used as a statistical tool and results are expressed as mean \pm SD.

3. RESULTS AND DISCUSSION

3.1. Isolation of PSB

Microbial phosphate solubilization is important in promoting the plant growth. Several researchers have reported that inoculation of PSM in fields and greenhouse conditions promotes plant growth [23,34]. Bacterial ability to multiply fast compared to fungi and actinomycetes leads to dominate the phenomena of phosphate solubilization in soil [35].

Based on morphologically distinct colony characters on nutrient agar, forty nine bacterial isolates were selected and screened for P solubilization on NBRIP agar medium. Thirteen isolates solubilized TCP, under plate (Fig. 1) and broth assay. The highest P solubilization index was observed in MGnB10 that is 2.8,

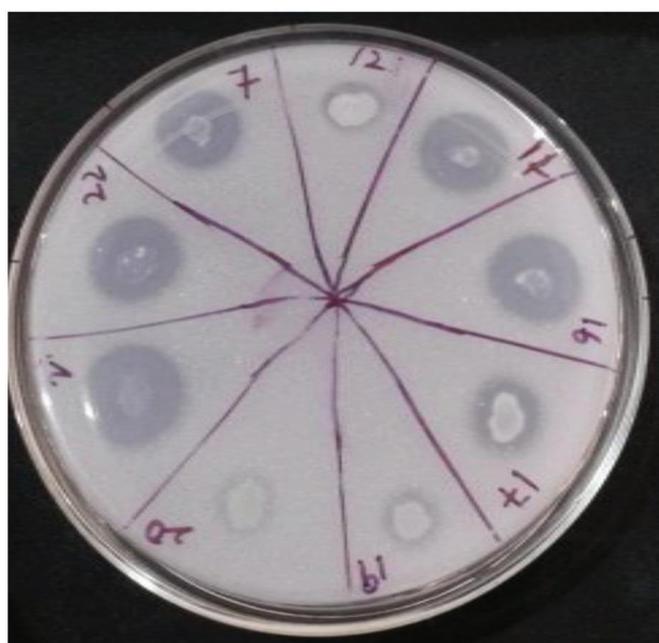


Figure 1: Solubilization of TCP on NBRIP agar medium by bacteria.

followed by MRB4 i.e. 2.6, but their respective P solubilization varied as MGnB10 solubilized 372 µg/ml, MRB4 solubilized 389 µg/ml, highest among all thirteen isolates. Earlier, research study done by Gupta et al. [36], Nautiyal [20], also supports our findings. Our results also reveals similarity in reduction of pH by PSB isolates, however there was variations in amount of P solubilized. For MMzB5 and MRB16 final pH was observed as 4.38, however solubilized P was estimated as 334 and 315 µg/ml, (Table 1) these results are very well corroborated with the findings of Gulati et al. [37] and Sujatha et al. [38], it indicates that decreasing pH due to organic acid production [39] is not the single factor for P solubilization. H⁺ translocation, chelating agents, production of inorganic acids etc., also take their share of solubilizing P in soil [4,40].

3.2. Plant Growth Promoting Activities of PSB Isolate

Synthesis of biocontrol substances and plant growth regulators by PSM in addition to P solubilization enhances its applications as efficient bioinoculants [41]. Out of 13 PSB isolates, MMzB5, MRB4, and MRB16 were positive for all PGP activities (Table 2).

Out of 13, 12 isolates produced IAA, highest concentration 65 µg/ml produced by MRB4, lowest concentration was produced by MMzB12 20 µg/ml, IAA negative isolate is MGnB11. About 80% of rhizosphere microflora also owns the ability to produce auxins and our results are very well supported by the work of Patten and Glick [42]. All isolates were found to be positive for ammonia production, with variation in the intensity of yellow- brown color, six out of thirteen PSB isolates developed dark brown color, while other showed moderate ammonia production. Nine PSB isolates were positive for HCN production, only MRB4 showed strong HCN activity; others were moderate or weak producers. The role of IAA production [23,43], ammonia production [44,45], and HCN [46], and their subsequent impact on overall growth of plant have been reported. Nine PSB isolates were positive for protease enzyme and only six isolates showed zone of Congo red discoloration on cellulose Congo red agar media, indicates production of cellulase enzyme [15].

Table 1: Solubilization of TCP, qualitative and quantitative assay and pH change by PSB isolates.

S.No.	PSB isolates	Solubilization index (SI)	TCP Solubilization µg/ml	pH
1.	MMzB3	2.2 ± 1.10	326 ± 2.3	4.72 ± 1.45
2.	MMzB5	2.4 ± 1.47	334 ± 2.21	4.38 ± 0.86
3.	MMzB11	2.6 ± 2.39	333 ± 2.0	4.56 ± 0.79
4.	MMB12	1.8 ± 2.18	215 ± 3.95	4.89 ± 1.16
5.	MRB4	2.6 ± 0.85	389 ± 2.14	3.92 ± 0.32
6.	MRB7	1.8 ± 1.68	240 ± 1.82	4.16 ± 1.22
7.	MRB15	2.1 ± 1.54	244 ± 2.08	5.22 ± 1.14
8.	MRB16	2.6 ± 2.41	315 ± 1.66	4.38 ± 1.38
9.	MGnB3	2.3 ± 2.50	317 ± 2.58	3.76 ± 1.09
10.	MGnB9	2.6 ± 1.29	342 ± 1.53	4.13 ± 0.29
11.	MGnB10	2.8 ± 1.12	372 ± 1.05	4.34 ± 1.49
12.	MGnB11	2.3 ± 2.33	248 ± 1.62	4.81 ± 1.32
13.	MGnB16	1.8 ± 1.68	253 ± 3.61	5.06 ± 0.45

PSB isolates MMzB12, and MGnB11, do not show any antifungal activity, best among all is MRB4 (Fig. 2), other PSB isolates response towards *M. phaseolina* is good but against *S. rolfii* very poor [30]

3.3. Plant Growth Promotion Studies on Maize

After PGP activities, PSB isolates were tested on maize (*Z. mays*), MRB4 showed maximum seed vigor index (4465) (Table 3) followed by MMzB5 (4232), our findings are similar with the work of Pacome et al. [12,23,31,47]. These results suggest that treatment with PSB is beneficial as it improves the growth of maize plant due to its ability to produce plant growth regulators in addition to solubilize phosphates [48].

3.4. Biochemical Identification

After performing Gram staining, motility and other biochemical test, PSB isolates were identified. MMzB5, MRB4, MRB7,MRB15,MGnB3, and MGnB16 belongs to the genus *Bacillus* (6), MMzB3, MMzB11, MRB16, MGnB9, and MGnB10 are from genus *Pseudomonas* (5), MMzB12 and MGnB11 from *Klebsiella* (Table 4). *Bacillus*, and *Pseudomonas* are considered as dominant genera of bacteria, present study is supported by the work of many researchers [1].

3.5. Stress Tolerance Studies

Agricultural needs are severely affected due to abiotic stress, such as temperature, pH, drought and salinity, an unavoidable problem which influenced the growth of plants. Microorganisms have

Table 2: Plant growth promoting traits in PSB isolates.

Isolates	IAA $\mu\text{g/ml}$	Ammonia	HCN	Protease	Cellulase	Antifungal activity	
						<i>M. phaseolina</i>	<i>S. rolfii</i>
MMzB3	40 \pm 1.41	+++	+	-	-	-	+
MMzB5	40 \pm 0.23	+++	++	++	++	++	+
MMzB11	28 \pm 2.53	+++	++	++	-	++	+
MMzB12	20 \pm 2.86	++	+	-	-	-	-
MRB4	65 \pm 2.53	+++	+++	+++	+++	+++	++
MRB7	32 \pm 2.45	++	-	++	-	-	+
MRB15	25 \pm 1.24	++	++	++	+	+	-
MRB16	42 \pm 2.68	+++	++	++	++	++	++
MGnB3	30 \pm 1.24	++	-	++	+	++	+
MGnB9	28 \pm 0.05	++	-	-	-	+	+
MGnB10	38 \pm 1.87	+++	++	++	++	++	-
MGnB11	-	++	-	-	-	-	-
MGnB16	30 \pm 1.09	++	++	++	-	+	+

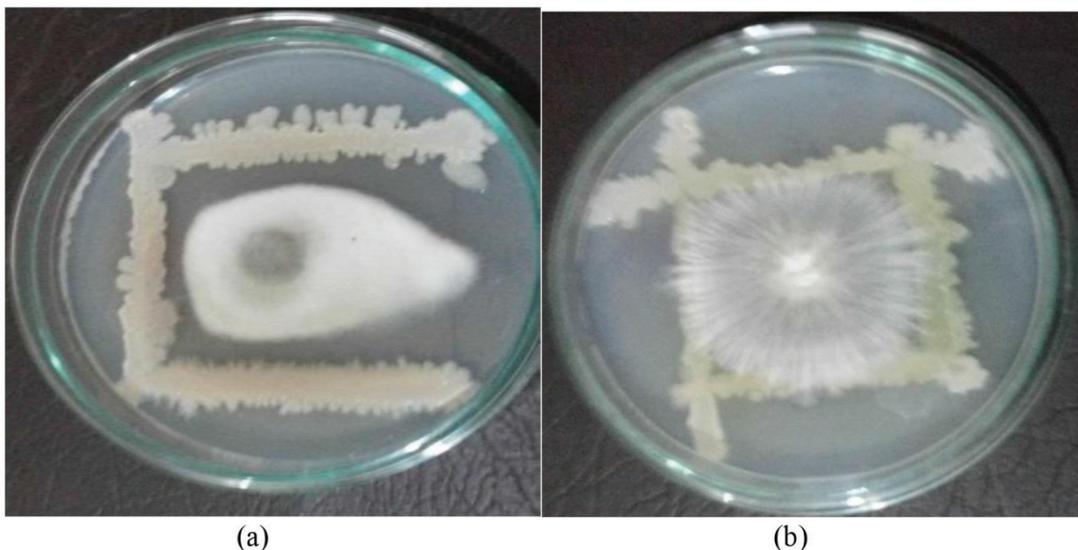


Figure 2: Antifungal activity of MRB4, against (a) *M. Phaseolina* and (b)

Table3: Effect of PSB isolates on *Z. mays*.

Isolates	Root length	Shoot length	Germination	Seed vigor
Control	9 ± 0.96	29 ± 1.36	83 ± 0.78	3,154 ± 1.41
MMzB3	10 ± 1.82	31 ± 1.66	92 ± 2.1	3,772 ± 0.23
MMzB5	12 ± 0.78	34 ± 1.19	92 ± 2.21	4,232 ± 1.6
MMzB11	11 ± 2.3	33 ± 2.21	94 ± 1.82	4,136 ± 2.38
MMzB12	9 ± 1.78	30 ± 2.34	89 ± 1.17	3,471 ± 2.21
MRB4	12 ± 1.53	35 ± 1.62	95 ± 1.05	4,465 ± 1.04
MRB7	10 ± 2.44	30 ± 2.21	92 ± 2.21	3,680 ± 2.53
MRB15	11 ± 1.94	33 ± 1.53	92 ± 1.66	4,048 ± 1.91
MRB16	11 ± 2.11	34 ± 1.19	92 ± 1.82	4,140 ± 1.73
MGnB3	10 ± 1.53	30 ± 1.62	89 ± 1.07	3,560 ± 1.24
MGnB9	10 ± 2.51	29 ± 2.23	85 ± 2.62	3,315 ± 2.67
MGnB10	11 ± 1.19	33 ± 1.66	94 ± 1.94	4,136 ± 1.91
MGnB11	9 ± 2.11	29 ± 2.40	85 ± 1.72	3,230 ± 1.91
MGnB16	11 ± 2.54	32 ± 2.64	92 ± 2.40	3,956 ± 2.53

Table 4: Morphological and biochemical characteristics of PSB isolates.

Characteristics	MMzB3	MMzB5	MMzB11	MMzB12	MRB4	MRB7	MRB15	MRB16	MGnB3	MGnB9	MGnB10	MGnB11	MGnB16
Margin	Entire	Wrinkled	Entire	Entire	Irregular	Irregular	Irregular	Entire	Entire	Entire	Entire	Entire	Entire
Elevation	Raised	Flat	Flat	Flat	Flat	Convex	Convex	Flat	Flat	Convex	Convex	Raised	Convex
Consistency	Mucoid	Mucoid	Mucoid	Mucoid	Dry	Dry	Dry	Mucoid	Dry	Mucoid	Mucoid	Mucoid	Dry
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Translucent	Opaque	Translucent	Translucent	Opaque	Opaque
Grams nature	Negative	Positive	Negative	Negative	Positive	Positive	Positive	Negative	Positive	Negative	Negative	Negative	Positive
Motility	+	+	+	-	+	+	+	+	+	-	-	-	+
Indole	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl Red	-	-	-	-	-	-	-	-	-	-	-	-	-
Voges Proskauer	-	+	-	+	+	+	+	-	+	-	-	-	+
Citrate	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	-	+	-	-	-	-	+	-	+	+	-	-
H ₂ S production	-	-	-	-	-	-	-	-	-	-	-	-	-
Starch Hydrolysis	-	+	-	-	+	+	+	-	+	-	-	-	+
Urea Hydrolysis	-	+	-	+	+	+	+	-	+	-	-	-	+
Gelatin Hydrolysis	+	+	+	-	+	+	+	+	+	+	+	-	+
Nitrate Reduction	+	+	+	+	+	+	+	+	+	+	+	+	+
Dextrose	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	-	+	-	-	+	+	+	-	+	-	-	-	+
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol	-	+	-	-	+	+	+	-	+	-	-	-	+
Xylose	+	+	+	-	+	+	+	+	+	+	+	+	+
Remark	<i>Pseudo</i>	<i>Bacillus</i>	<i>Pseudo</i>	<i>Klebsiella</i>	<i>Bacillus</i>	<i>Bacillus</i>	<i>Bacillus</i>	<i>Pseudo</i>	<i>Bacillus</i>	<i>Pseudo</i>	<i>Pseudo</i>	<i>Klebsiella</i>	<i>Bacillus</i>

Positive = (+), Negative = (-), *Pseudo*= *Pseudomonas*.

unique property of adapting to changing environmental condition [49,50]. Successful utilization of these organisms in stressed ecosystems depends on their ability to resist and multiply under unfavorable environmental conditions, such as high temperatures, salt stress, heavy metal toxicity, mineral deficiency etc.

Based on the results of PGP activities and effect of PSB isolates on seedlings growth and seed germination, five best PSB isolates (*Bacillus* spp. MMzB5, MRB4, *Pseudomonas* spp. MMzB11, MRB16, and MGnB10) (Fig. 3) were screened for their P solubilization potential under various abiotic stress such as temperature (45°C), pH (9), salinity (8%) and drought (-0.49 Mpa). The isolates seemed generally well adapted to the environments, all five isolates demonstrated diverse levels of P solubilization. *Bacillus* sp. MRB4 was the best isolate among all as it shows P solubilization under all abiotic stressed condition followed by other *Bacillus* sp. MMzB5. As *Bacillus* spp possess the ability to produce endospore, it can combat against high temperature, salinity, and drought conditions more strongly compared to *Pseudomonas* isolates [30].

To be metabolically active and proliferate, microbes require a suitable temperature, but due to change in soil temperature, their physiological activities may get affected. In tropics, during

summer, ambient temperature, and soil temperature vary between 35°C and 45°C, the ability of the organism to survive and grow at higher temperature in the soil as well as in the inoculant packets during transport and storage is one of the most desired characteristic. *Bacillus* spp. MRB4 solubilizes 193 µg/ml of P, followed by MMzB5 122 µg/ml. Although high temperature affect the P solubilization ability of the PSB isolates, however the inoculation of thermotolerant PSB isolates, improved the growth of Mung bean (*Vigna radiata*) as stated by Gaind and Gaur [51].

According to a study conducted by International Crops Research Institute for the Semi-arid Tropics in 2015, it was revealed that due to digging of bore well, salt is accumulating in soil, which is increasing soil pH [52], results in poor growth and survival of PSB [53]. All PSB isolates showed the P solubilization at alkaline pH 9, 181 µg/ml solubilized by *Bacillus* spp MRB4, followed by *Pseudomonas* spp. MRB16, MMzB11 solubilizes 141 and 106 µg/ml.

Salinity adversely affects the plant growth, development [54], and equally affects the survival and proliferation of microbes in soil and rhizosphere [55]. *Bacillus* spp. MRB4 solubilizes 124 µg/ml under 8% salinity stress, 385 µg/ml in control, other isolates did not show promising solubilization under salinity stress. In general, the amount of P released is found to

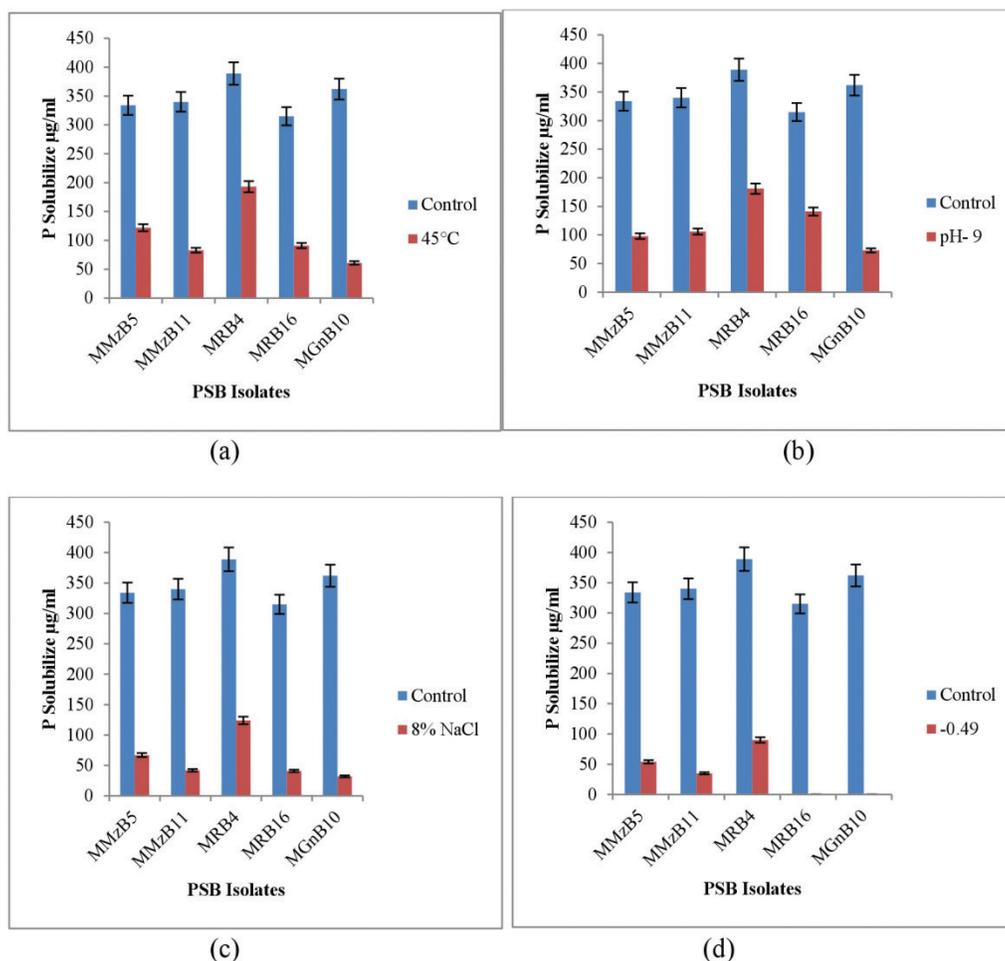


Figure 3: P solubilization under various abiotic stresses by five selected PSB isolates. (a) Temperature (45°C), (b) pH (9), (c) Salinity (8%), and (d) Drought (0.49 Mpa). Results represent the mean of three replicates \pm standard deviation.

decrease with increase in salt concentration and our results are in accordance with the work of Mohan et al. [56]. P solubilization was more in control than in medium supplemented with sodium chloride (NaCl).

In India, agricultural productivity is mostly limiting due to drought stress, which influences plant water relations, damaging plant growth. Inoculation of plants with drought tolerant beneficial microbe, improves the growth and tolerance of drought in plant as suggested by Ali et al. [19]. Out of five PSB isolates, three solubilizes P (MMzB5, MMzB11, and MRB4) under -0.49 Mpa

and considerable solubilization was observed with isolate MRB4 90 µg/ml.

3.6. Molecular identification

Based on the potential to solubilize TCP under various abiotic stressed conditions and plant growth promoting traits, best isolate, namely, MRB4 (*Bacillus* spp.) was selected for molecular identification by 16S rRNA sequencing, showed highest similarity to *Bacillus subtilis*, sequence is submitted in NCBI GenBank with accession number MK611797 (Fig. 4).



Figure 4: Evolutionary relationships of taxa the evolutionary history was inferred using the neighbor-joining method [57]. The optimal tree with the sum of branch length = 0.31480504 is shown. Next to the branches, the percentage of replicates in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown [58]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to deduce the phylogenetic tree. Kimura 2-parameter method was used for computing evolutionary distances [59] and are in the units of the number of base substitutions per site. 32 nucleotide sequences were analyzed. Positions containing gaps and missing data were eliminated. In the final dataset their were total 1,125 positions. MEGA7 was used for conducting evolutionary analyses Kumar et al. [60]. Only bootstrap values $\geq 70.0\%$ are shown.

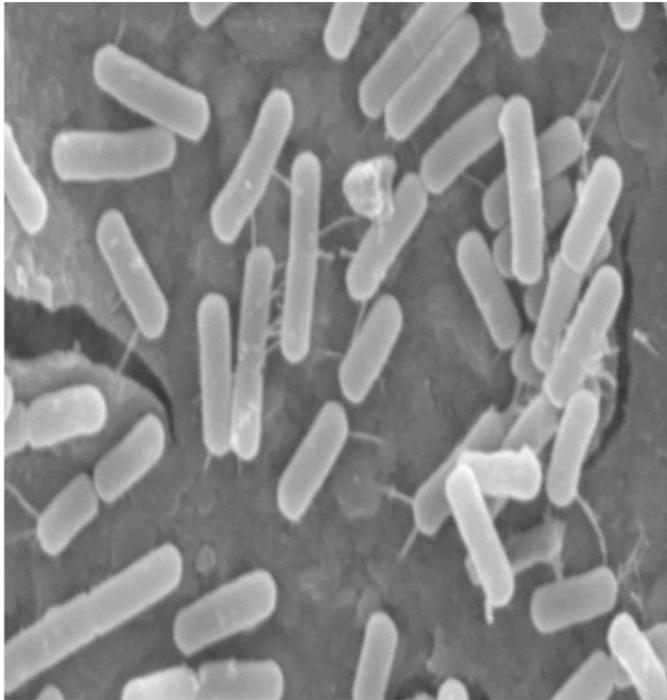


Figure 5: SEM image *B. subtilis* MRB4.

3.7. Scanning of electron microscopic image of *B. subtilis*

To know the surface morphology of efficient PSB isolate *B. subtilis* MRB4 better, Scanning electron image was taken in Department of Physics Osmania University, Hyderabad. Instrument used was Zeiss, with higher magnification. Rod shaped cells were observed (Fig. 5).

4. CONCLUSION

Bacillus subtilis MRB4, isolated from the rice field of Mahbubnagar, a district of Telangana state, India, is a promising plant growth promoter and a potential P solubilizer under stress (es) *in vitro*. Hence further evaluation of this bacterial strain is needed in greenhouse and field condition.

5. ACKNOWLEDGMENTS

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6. CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

7. FINANCIAL SUPPORT & SPONSORSHIP

None.

8. AUTHORS' CONTRIBUTION

Authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

9. LIST OF ABBREVIATIONS

µg	Micro gram
HCN	Hydrogen cyanide
IAA	Indole acetic acid
NBRIP	National Botanical Research Institute Phosphate
P	Phosphorous
PSB	Phosphate solubilizing bacteria
PSM	Phosphate solubilizing Microorganisms
SEM	Scanning Electron Microscope
TCP	Tri calcium phosphate

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