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Research Article

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Detection of phosphate solubilizing bacteria from rhizospheric soil of agricultural crops in Erode district

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Abstract : Phosphorus is a second key plant nutrient and the 'kingpin' in Indian agriculture, occupies an exclusive position both in conventional as well as in alternative agriculture. However, the phosphate solubilizing bacteria (PSB) converts the insoluble phosphate into biologically available form through the production of organic acids. Hence, the present study explored the presence of PSB from the Karavalasu agricultural field, Sivagiri, Erode District. The percentages of bacterial solubilization were 14.40 % to 60.70 % in non rhizospheric and rhizospheric crops adhering to root soils respectively.Out of 14 morphologically distinct isolates of PSB, two isolates (PSB8 and PSB11) displayed high P solubilization efficiency on solid medium amended with tricalcium phosphate $Ca_3(PO_4)_2$ as the insoluble source (0.2 %). They were further characterized and continued with identification used by Picovskaya's selective medium. The selective medium was incubated for 48 hours at a temperature of 37°C. The characterization was done for two strains by looking at the colony morphology and biochemical properties. The isolates showed the optimum pH for growth of the organism was pH 7 and the optimum temperature was 37 °C. Two strains exhibited significant release of P at the concentrations of 1.96 and 2.26 mg/l by PSB – 8 and PSB – 11 respectively on 10th day of incubation in broth medium amended with $Ca_3(PO_4)_2$. The strains released acids as evident by decline in pH of the broth medium. They also secreted IAA with the maximum of 17.06 mg/l by PSB - 8 with $Ca_3(PO_4)_2$ as a source of P. The potential strains for P solubilization were identified using 16S rRNA as *Bacillus megaterium* and *Pseudomonas aeruginosa* for further application as bioinoculants to agricultural fields. Finally we afford some indication that the use of Phosphate solubilising microorganisms will support sustainable agriculture and conclude that this knowledge is ready for commercial utilization in various regions worldwide.

Keywords: Rhizospheric soil, phosphate solubilizing bacteria, indole-3 acetic acid, bioinoculants

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1. INTRODUCTION

Phosphorous (P) plays an irreplaceable role in the ecosystem by contributing in most portion of energy metabolism, kinase regulation, nucleic acid and protein synthesis.[1]Phosphorus has long been considered the second most limiting nutrient for plant growth in terrestrial ecosystems after nitrogen.[2]The total phosphorus content in the soil of arid zones in India ranges from 560 to 900 kg/ha but the available phosphorus in soil is only 15 - 25 kg/ha, it's quite low comparatively with total phosphorus. The release of insoluble and fixed forms of phosphorus is an imperative feature for increasing the P accessibility in soil.[3] Phosphorus is an important plant macronutrient that plays a momentous role in plant metabolism, ultimately reflected on production of crop yields. It is significant for the functioning of key enzymes that regulate the metabolic pathways. It is estimated that about 98% of Indian soils contain insufficient amounts of available phosphorus, which is necessary to support and enhance the maximum plant growth. The uptake of phosphorus by the plant root is only a small fraction of what is actually added as phosphate fertilizer in soil. Phosphorus deficiency is widespread and phosphorus fertilizers are required to maintain crop production. When it is added to the soil in the form of phosphatic fertilizer, only a small portion is utilized by plants for biomass production and crop improvement.[4] In India, the majority of the phosphorus isprovided in the form of chemical fertilizers which decreases the fertility of soil after a long period of time. In nature, a wide range of microbial biosolubilization mechanisms exist which are necessary to maintain the global cycle.[5] A large portion of soluble inorganic phosphate is applied to the agricultural soil as chemical fertilizers are rapidly immobilized soon after application and becomes unavailable to plants.[6] Plants can absorb phosphate only in soluble form. The transformation of insoluble phosphate into soluble form is carried out by a number of microbes present in the soil.[7,8] Facility to dissolve phosphate by microorganisms is a vital trait, carried out by a huge number of saprophytic bacterial and fungal microorganisms that are associated with phosphate nutrients for plants. The naturally occurring rhizospheric phosphate solubilizing microorganisms (PSM) have first been reported in 1903.[9] The soil microorganisms are actively involved in the immobilization processes that arrest deposition of phosphorus in land by solubilization and also change the phosphorus cycle in soil.[10,11] Generally the soil microorganisms are effective in releasing both organic and the inorganic phosphorus from the total phosphate soil through a process of dissolution.[12,13] Most of the soil phosphate is usually present as insoluble metal chelates.[14] In this circumstance, microbial solubilization of soil insoluble phosphates into soluble forms are considered as an important process in natural and agricultural retaining forms of phosphorus. It also plays an important role in biogeochemical phosphorus cycling in both terrestrial and aquatic environments.[15] However, the reports of phosphate solubilizing bacteria in agricultural fields are high and the present study was made to isolate and enumerate the phosphate solubilizing bacteria associated with rhizospheric soil of two crop zones in relation to physico-chemical characteristics of the soil.

2. MATERIALS AND METHODS

2.1. Collection of Soil Samples

The soil samples adhering to the root system of two crop species viz., *Saccharum officinarum, Coriandrum sativum* and non rhizospheric soil sample acted as control were collected carefully using the vertical corner from a selected agricultural field in Karavalasu, Sivagiri, Erode District (Lat. 11° 0' 34.92"; Long. 77° 41' 45.7074") (Figure 1), which is situated in the southern part of India. Samples were randomly collected three times from the rhizosphere of young plants at a depth of 10 –20 cm and from the non crop zone. The collected soil samples were stored in polythene bags aseptically and maintained at the laboratory for further study.

Source: https://goo.gl/maps/HZ7E66SGA9GtaFGaA

Fig 1: A pinpointed areas clearly showing the location of the study sites

2.1.1. Physico–Chemical Characters of Rhizospheric Soil Samples

The texture of soil samples were calculated by pipette method.[16]The temperatures of three soil samples were measured by using a mercury centigrade thermometer with 0.5 ˚C accuracy. The pH of soil samples were measured in the diluted samples at 1: 2.5 soil and water ratio. The pH in the solution was measured using an Elico pH meter, calibrated with standard buffer solution prior to use. Electrical conductivity (EC) was measured in the rhizospheric soil samples prepared for analyzing the total amount of soluble salts present in the soil. The electrical conductivity (EC) is expressed as units to be written properly dsm^{-1}. The EC of the soil extract was determined by using a Systronic EC meter (digital) in a soil water suspension of 1: 5 ratios.[16] Soil moisture is the total amount of water content in the soil on its dry weight basis.[17] The water holding capacity of the soil was followed by Piper.[18] The bulk density and porosity of the soil samples were followed by Black method.[19]

2.2. Nutrient analysis of Rhizospheric soil

2.2.1. Macronutrient analysis

2.2.1.1. Determination of available Nitrogen in soil[20]

Twenty gram of soil was placed in a distillation flask to this 20 ml of water, 100 ml of 0.32 % $KMnO_4$ solution and 100 ml of 2.5 % NaOH were added. 20 ml of 2 % boric acid was pipette out from the conical flask to these 2–3 drops of mixed indicator was added and dipped the end of the delivery tube into it. Ammonia gas from the distillation flask was distilled and collected in boric acid solution. Distillation was continued till the evolution of ammonia ceases completely (test by bringing a moist red litmus paper near the outlet of the condenser, which will turn blue as long as ammonia was being evolved). Titrated against N/50 H_2SO_4 and noted the volume of H_2SO_4 used. The end point is reached when the colour changes from pink to blue.

2.2.1.2. Phosphorus estimation [21]

Ten gram of soil sample was digested with ternary acid mixture (10 ml conc. HNO_3, 1ml conc. H_2SO_4 and 4 ml 60 % $HClO_4$) until it became colourless. After digestion, it was extracted with distilled water and then filtered. The volume of the extract was made upto 100 ml. Ten ml of the solution was taken in 50 ml volumetric flask and 2 drops of dinitrophenol was added. To this solution, 2 ml of sulphomolybdic acid was added and the volume was made upto 50 ml. The solution was transferred to 100 ml conical flask and added with 3 drops of chlorostanous acid with intermittent shaking. After 5 minutes, optical density of the developed colour was determined in a photoelectric colorimeter (Systronics - 116) at 660 nm. The concentration of phosphorus in the test solution was calculated from a standard graph developed from potassium dihydrogen phosphate (KH_2PO_4) solution of different concentrations. The result is expressed on per cent dry weight basis.

2.2.1.3. Determination of available Potassium in soil [22]

Five gram of soil was taken in a 150 ml conical flask. To this 25 ml of neutral N NH_4OAC solution was added. The contents of the conical flask were agitated with an electric shaker for 5 minutes and filtered. The filtrate was subjected into the atomizer of the flame photometer (Esico - 1381) at 100 ppm of which has been set with 40 ppm K solution and the results were noted. These noted readings were plotted on the graph for calculating the standard curve, which will give the K concentration in the soil. From this concentration measurement, the amount of K in the sample was calculated.

2.2.1.4. Organic carbon estimation[16]

A weighed amount of oven dried soil (1 g) was taken in a 500 ml Erlenmeyer flask and added with exactly 10 ml of normal potassium dichromate solution and 20 ml of conc. H_2SO_4 successively. The flask was shaken for 2-3 minutes and allowed to 45 stands on a sheet of asbestos for 30 minutes. After 30 minutes, the contents were diluted with 200 ml distilled water and added with 10 ml phosphoric acid and 1 ml diphenylamine indicator until the colour of the mixture flashes to green. An excess of 0.5 ml of dichromate was added and the titration was completed by adding sulphate solution drop by drop until the last traces of blue colour disappeared.

2.2.2. Micro Nutrient Analysis

The micronutrients viz., iron, manganese, zinc and copper were estimated by using Atomic Absorption Spectrometer (AEI - 5310).[23]

2.3. Seclusion Of Bacterial Isolates

Rhizosphere soil samples were collected during the rainy season from three different zones (*Saccharum officinarum* and *Coriandrum sativum* and non crop zones) and brought to the laboratory immediately for analyses within 3 hours. Soil samples were collected in sterile polythene bags using a sterile spatula and shade-dried to a constant weight. Before that the plant roots and other debris were removed from the soil samples. Then the sediments were ground and sieved (mesh size of 2 mm) with filter. Then the soil samples were stored at 5 ˚C for further study. A known weight of rhizosphere soil (10 g) was aseptically weighed and transferred to a stoppered (150 ml) sterile conical flask containing 100 ml of sterile diluent. The sediment-diluent mixture was agitated by means of mechanical shaking for about 15 minutes which served as stock solution for later bacteriological examination. For isolation of P solubilizing bacteria, the Picovskaya's medium was prepared for 1 litre.[24] An aliquot of (10^{-2}, 10^{-4}, 10^{-6}) decimal dilutions were inoculated on Picovskaya's medium by pour plate technique and incubated at 37 ◦C for P solubilizing bacterial counts. For plating, one millilitre of the serially diluted samples of soil was pipetted out into sterile petri-dish. Sterile media were then poured into dishes aseptically and swirled for through mixing and allow for solidify. After solidification, the plates were incubated at 37°C. . All the determinations were carried out in duplicates. After the incubation period of 2 days for P solubilizing bacteria, colonies were counted for intermittent dilutions (10^{-2}, 10^{-4}, and 10^{-6}). The counts are expressed as colony forming units (cfu) per gram of the soil. Inorganic P solubilizing bacteria were identified by clear solubilizing zones that were formed around their colonies at the end of the third day of incubation and formed as creamish colonies. From the isolates, larger halo zone (2 cm) producing isolates were selected for further study. Isolates showing phosphate solubilizing ability were spotted inoculated at the centre of the Pikovskaya's plate. The qualitative as well as quantitative analyses of phosphate solubilizing activity of the selected isolates were conducted by plate screening method and broth culture method.

2.4. Colony measurement

The bacterial colonies that appeared on the Pikovyskya's agar media were measured successively after 24 hours up to 2 days of incubation. The microbial populations were expressed as colony forming units per gram dry soil for bacteria.

$$\text{Microbial (bacterial) population} = \frac{\text{Mean number of colonies per plate}}{\text{Volume of diluted sample plated}} \times \text{Dilution factor}$$

2.5. Percentage of phosphate solubilizing bacteria[25]

For calculating the percentage of PSB the following formula was used.

$$\text{PSB (\%)} = \frac{\text{Total number of Phosphate solubilizing bacteria}}{\text{Total number of bacteria}} \times 100$$

2.5.1. Enumeration and isolation of P solubilizing bacteria

Enumeration of phosphate solubilizing bacteria (PSB) present in the soil samples was done by adopting plate count methods in the dilutions of 10^{-2}, 10^{-4} and 10^{-6}. Tricalcium phosphate $Ca_3(PO_4)_2$as the insoluble source of phosphate used at 0.2 % in the Pikovskaya's agar medium helped in the enumeration of bacteria. The plates were incubated for three days at 37˚C in an incubator. The colonies that exhibited the clearing zone were considered as P solubilizers. The clear-zone forming organisms were counted, isolated and purified. The 14 most predominant and morphologically distinct bacterial colonies were selected for qualitative assay and were designated as PSB - 1, PSB - 2uptoPSB - 14.

2.5.2. Estimation of phosphate solubilizing potential of the isolates

In the qualitative study, all the 14 bacterial isolates were tested for solubilization efficiency by means of plate assay using Pikovskaya's medium containing 0.2 % of tricalcium phosphate as insoluble source. The plates were incubated at 37 ˚C for 48 hours. Isolates showing phosphate solubilizing ability were spot inoculated at the centre. By measuring the diameter of the clear zone and colony growth, P solubilization efficiency was tested.[26]

$$\text{PSE} = \frac{\text{Solubilization diameter}}{\text{Diameter of the colony growth}} \times 100$$

Based on the results of the plate assay, two isolates (PSB - 8 and PSB - 11) which showed the best solubilization of P was marked and identified using molecular marker 16S rRNA. They were initially matched with morphological characteristics, gram staining (HansChristian Gram, 1884), motility, IMViC test including catalase, oxidase, urease, gelatinase, protease, lipase and carbohydrates utilization tests including amylase, triple sugar iron**,** sucrose, glucose, lactose fermentation, maltose, fructose and galactose. The isolated bacterial strains were identified using standard preliminary test and biochemical tests as listed in the Bergey's Manual of Determinative Bacteriology.[28] Later they were subjected to further experimental studies such as quantitative estimation (broth assay), influence of the isolates on pH (Jenway-3510) of the medium.[29] and production of IAA. The PSB strains were streaked on Pikovskaya's medium and kept at different temperatures 5, 25, 35 and 50 °C) and recorded the optimum growth condition.

2.5.3. Quantitative estimation of P solubilizing potential of the isolates (broth assay)

The two selected bacterial isolates (PSB - 8 and PSB - 11) were screened to find out the amount of P solubilized in the broth by growing them in 100 ml Erlenmeyer flasks containing 50 ml of Pikovskaya's broth supplemented with 0.2 % tricalcium phosphate as the insoluble source $Ca_3(PO_4)_2$. Appropriate uninoculated controls were maintained. All the treatments were replicated. The bacterial cultures were withdrawn after the sixth, eighth and tenth day of incubation at 37 ˚C for the estimation of soluble P. The bacterial cultures were centrifuged at 15,000 rpm for 20 minutes and the supernatant was passed through 0.2 µm membrane filter so as to obtain the culture filtrate containing only the soluble forms of metal.[30] Then the sample was fed to an Inductively Coupled Plasma / Optical emission Spectrometer (ICP – OES) to find the concentration of available P present in the sample.

2.5.4. Influence of P solubilizing bacteria on pH of the growth medium

The selected isolates (PSB - 8 and PSB - 11) were inoculated in the flasks containing 50 ml of sterilized Pikovskaya's medium supplemented with 0.2 % of tricalcium phosphate as the insoluble source $Ca_3(PO_4)_2$. An uninoculated sample was also maintained. The samples were drawn on the sixth, eighth and tenth day after incubation. The bacterial cultures were centrifuged at 15,000 rpm for 10 minutes and filtered using Whatman No. 42 filter paper. The pH of the PSB culture filtrates were measured using a pH meter (Elico).

2.5.5. Quantitative estimation of Indole-3 Acetic Acid by P solubilizing bacterial isolates

The selected bacterial isolates were tested for their ability to produce IAA by inoculating the flasks containing 50 ml of Pikovskaya's medium supplemented with 0.2 % tricalcium

phosphate as the insoluble source $Ca_3(PO_4)_2$. Another set devoid of P was also maintained. All the treatments were amended with 0.1 % tryptophan and incubated for seven days. The quantities of IAA produced by the organisms were estimated.[31]

2.6. *Molecular Identification*

The 16S rRNA amplification was done by using universal Forward primer 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and Reverse primer 1492R (5'-GGT GTT TGA TTG TTA CGA CTT-3') to identify the bacterial species. The PCR reactions were run by using 50 µl reaction mixture containing 1µl of RNA extract as a template, 5 mM of each primers, 25 mM of $MgCl_2$, 2 mM of dNTPs, 1.5 U of Taq polymerase and buffer recommended by the manufacturer (GCC Biotech, India). After initial denaturation for 10 minutes at 96°C, 35 cycles of reaction was performed consisting of denaturation at 96 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 2 min and final extension at 72 °C for 7 minutes. The PCR products were purified and sequenced (Applied Biosystems). The phylogenetic tree was constructed using the neighbour – joining methods with the help of MEGA 6 software.[32]Bootstrap analysis was based on 1000 re-samplings. The 16S rRNA sequences of two strains were uploaded in the NCBI, coded with proper accession number.

3. STATISTICAL ANALYSIS

The significance of variations in phosphate solubilizing bacteria among the different soil samples were determined by subjecting the data to one-way analysis of variance (ANOVA) using SPSS (version 16.0). Means were compared with Duncan's multiple range test ($P<0.05$).[33]

4. RESULTS AND DISCUSSIONS

4.1. *Physico-chemical characteristics of soil samples*

Soil characteristics are one of the important environmental factors that directly affect fertility and crop growth. The geographical location of three different study sites, physico-chemical characters and distribution of minor and major elements in rhizospheric soil samples around the root system of two crop fields and non rhizosphere soil samples are shown in Table 1. The soil nature in study sites was sandy loam soil and possessed alkaline pH in non crop zone and *Coriandrum* zone with low P content, whereas in *Saccharum* zone it was 6.7 pH ranges. Alkaline pH is known to precipitate and accumulate the heavy metals.[34]

Table 1: Physico-chemical characteristics of different rhizosphere soil samples (NCZ – Non Crop Zone; **SZ** - *Saccharum* Zone; **CZ** - *Coriandrum* Zone)

Source	Geographical Location	Texture of soil	Soil Temperature	pH	EC (dSm^{-1})	Soil moisture content (%)	Water holding capacity (%)	Bulk density (g/ccs) (g/cm^3)	Soil porosity (%)
NCZ (Control)	11° 18' 57" N 77° 40' 16" E	Sandy loam	25°C	7.1	0.60±0.10	5.63±0.50	24.29±1.04	0.85±0.12	32.34±1.09
SZ	11° 4' 14" N 77° 46' 49" E	Sandy loam	26°C	6.7	0.56±0.11	7.23±0.30	25.41±0.72	1.26±0.63	44.29±1.55
CZ	11° 4' 12" N 77° 47' 2" E	Sandy loam	24°C	7.8	0.70±0.10	7.00±0.45	23.78±0.53	0.87±0.72	40.68±1.48

Source	N (kg/ha)	P (kg/ha)	K (kg/ha)	Organic carbon (%)	Fe (ppm)	Mn (ppm)	Zn (ppm)	Cu (ppm)
NCZ (Control)	1.92±0.04	0.49±0.02	1.15±0.24	0.33±0.07	15.72±0.51	1.44±0.10	3.80±0.08	1.27±0.04
SZ	1.25±0.20	0.75±0.08	2.93±0.04	0.43±0.06	16.88±0.07	4.01±0.24	4.58±0.08	1.96±0.06
CZ	1.76±0.06	0.63±0.02	1.79±0.02	0.37±0.03	13.27±0.18	1.85±0.07	5.59±0.07	1.92±0.04

Note: Values are expressed as mean ± SD (n=3)

The temperature of the non crop zone and *Coriandrum* zone was 24 - 25°C, whereas in *Saccharum* zone it was 26°C. The soil samples from the three study sites displayed electrical conductivity in the range of 0.56 ± 0.11 to 0.70 ± 0.10dSm^{-1} and the percent soil moisture was ranged between 5.63 ± 0.50 to 7.23 ± 0.30. The highest soil water holding capacity and bulk density in all the three sites were noted in *Saccharum* zone (25.41 ± 0.72 % and 1.26 ± 0.6 g/cm^3 respectively). The porosity of soil in the three sites was generally around 30 – 45 %. Similar findings were reported in earlier studies.[35]The available nitrogen content was higher in non rhizosphere soil (1.92 ± 0.04kg/ha) followed by 1.76 ± 0.06kg/ha in the *Coriandrum* zone (Table 1). The available phosphorus and potassium contents were higher in the *Saccharum* zone (0.75 ± 0.08 and 2.93 ± 0.04 kg/ha) respectively. Similarly, Robson and Pitman[36] analysed that the increasing availability of P in the growth medium can induce Zn deficiency in plants by altering soil and plant factors. In contrast, the total organic carbon was found to be low with a range of 0.33 % to 0.43 %, due to high biological activity as suggested.[37] This large variation in the distribution of PSB in different soils may be due to the differences in organic carbon content of the soil.[38] Micronutrients are required in very tiny quantities. Also, they are harmful when the available forms are present in the soil in higher amounts than the level that could be tolerated by plants. In the present study, the level of ferrous content was found to be dominant (13.27 to16.88 ppm) when compared to the significant level (>4.5) in all the rhizospheric and non-rhizospheric soil samples followed by zinc content, which ranged between 3.80 and 5.59 ppm. Adequate Zn nutrition is important in controlling the uptake of P by roots.[39] The manganese and copper content was similar more equal, however, a reverse trend was observed for *Saccharum* zone.

4.2. *Total P solubilizing bacteria (PSB) in the different rhizospheric soil samples*

The present study isolated 14 most predominant and morphologically distinct PSB strains from the soil samples. Further, based on the plate assay, 2 isolates which showed the best solubilizations of P were detected. The total P solubilizing bacteria (PSB) along with solubilizer of $Ca_3(PO_4)_2$ in the different rhizospheric soil samples are given in the

Table 2. The population was limited in all the soils which accounted for 11 to 17 % of the total heterotrophic bacterial counts depending on the soil type and limited phosphate source. The maximum PSB was found in the crop field of *Saccharum* (10.33 ± 0.33) in this condition $Ca_3(PO_4)_2$ as the insoluble source. Whereas in the non crop zone showed limited populations of PSB compared to rhizospheric crop zones. The lowest phosphate solubilizing total heterophilic bacterial percentage was noted in NCZ (4.67 ± 0.33). The values obtained from three study zones were statistically significant (P<0.05) different according to Duncan's Multiple Range Test.

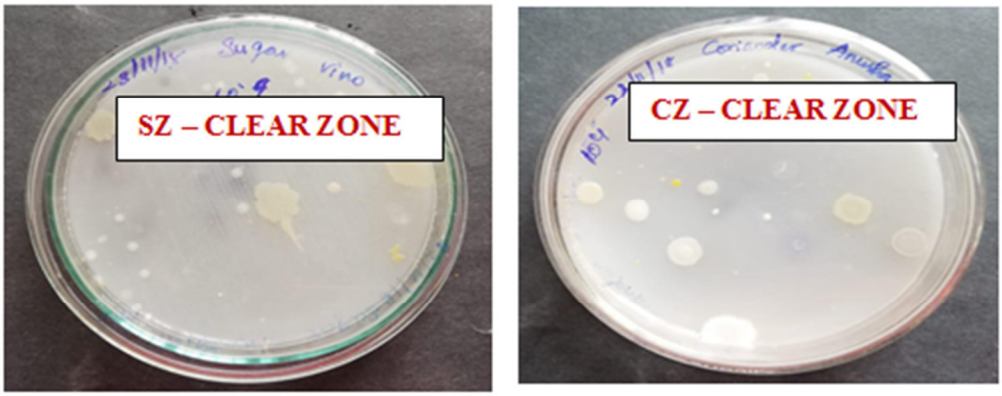
Table 2: The total PSB in the different rhizospheric and non rhizospheric soil samples (NCZ – Non Crop Zone; **SZ** - *Saccharum* Zone; **CZ** - *Coriandrum* Zone)

Source	Dilutions	THB (CFU x dilution 10^{-2},10^{-4}, 10^{-6}/g)	PSB (CFU x dilution /g)	Phosphate Solubilizing Bacteria (% of THB)
NCZ	10^{-2}	11.00 ± 0.32^{a}	3.67 ± 0.33^{ab}	28.00 ± 2.08^{b}
	10^{-4}	11.00 ± 0.36^{a}	3.00 ± 0.58^{b}	14.40 ± 0.99^{c}
	10^{-6}	13.00 ± 0.41^{a}	4.67 ± 0.33^{a}	37.50 ± 1.01^{a}
F=$df_{(2,8)}$		4.000 *	3.800*	63.888***
SZ	10^{-2}	15.67 ± 0.33^{b}	7.67 ± 0.33^{b}	51.33 ± 0.88^{a}
	10^{-4}	16.67 ± 0.33^{ab}	8.33 ± 0.33^{b}	44.00 ± 0.58^{b}
	10^{-6}	17.67 ± 0.33^{a}	10.33 ± 0.33^{a}	52.00 ± 0.58^{a}
F=$df_{(2,8)}$		9.000*	17.333***	40.923***
CZ	10^{-2}	14.67 ± 0.33^{ab}	7.00 ± 0.58^{b}	51.00 ± 0.58^{b}
	10^{-4}	14.00 ± 0.58^{b}	7.00 ± 0.58^{b}	52.00 ± 0.58^{b}
	10^{-6}	16.00 ± 0.58^{a}	9.67 ± 0.33^{a}	60.70 ± 0.91^{a}
F=$df_{(2,8)}$		4.000*	9.143*	43.439***

Note: THB - Total heterotrophic bacteria. Means ± S.E., in a column followed by the same letter (s) are significantly (P<0.05) different according to Duncan's Multiple Range Test. * ***Significant at 0.05 % level, ns - non significant.n=3

4.3. Qualitative estimation of P solubilizing potential of the isolates

In the qualitative study, the bacterial strains were tested using plate assay. The 14 bacterial isolates were inoculated in the Picovskaya's selective media amended with different $Ca_3(PO_4)_2$ as the insoluble source at 0.2 %. The solubilization efficiency of the isolates was calculated by measuring the diameter of the colony growth and the solubilization zone (Plate 1). Phosphate solubilizing potential varied with each isolate and solubilization efficiency ranged between 129.9 % and 365.93 % depending on the P source used (Table 3). Among the isolates, PSB - 8 and PSB -11 showed highest dissolution zone and solubilizing efficiency inoculated with 0.2 % tricalcium phosphate ($Ca_3(PO_4)_2$) as the insoluble source. The formation of the halo zone is due to the movement of organic acids, secreted from PSB for P solubilization.[40]A similar study has found that *Pseudomonas aeruginosa* species exhibit higher clearing zone with zinc oxide as Zn source, whereas *Pseudomonas* species produces higher halo zone with ZnO and $ZnSO_4$.[41]



SZ – *Saccharum* Zone **CZ – *Coriandrum*Zone**

Plate 1: Zone of phosphate solubilization around the colony growth of PSB on PVK agar plate

Table 3: *In vitro* phosphate solubilizing potential of the bacterial isolates

Bacterial isolates	Dilution	PSE %
PSB - 1	10^{-6}	158.66 ± 0.88^{h}
PSB – 2	10^{-4}	148.1 ± 0.95^{i}
PSB - 3	10^{-6}	177.8 ± 0.87^{e}
PSB – 4	10^{-4}	140.6 ± 0.87^{j}
PSB – 5	10^{-6}	337.5 ± 0.75^{c}
PSB – 6	10^{-6}	136.73 ± 0.72^{k}
PSB – 7	10^{-6}	171.46 ± 0.86^{f}
PSB – 8	10^{-6}	340.5 ± 0.76^{b}
PSB – 9	10^{-4}	129.9 ± 0.78^{l}
PSB - 10	10^{-6}	137.63 ± 0.87^{k}
PSB – 11	10^{-6}	365.93 ± 0.98^{a}
PSB – 12	10^{-4}	180.56 ± 0.86^{d}
PSB – 13	10^{-6}	164.46 ± 1.25^{g}
PSB - 14	10^{-6}	131.36 ± 0.87^{l}
$F=df_{(2,8)}$		5.413**

PSE – Phosphate Solubilizing Efficiency.
Means ± S.E., in a column followed by same superscript letter between values did not differ significantly at 5% level by Duncan's Multiple Range Test. **Significant at 0.05 % level, ns - non significant.

4.4. Morphological characteristics of the selected bacterial isolates

First line of identification for the organism was done by gram's staining technique. The isolates appeared rod shaped gram positive (PSB – 8) and gram negative (PSB – 11) structure when viewed under both phase contrast and 100 X oil immersion microscopy. The colonies were creamish yellow and creamish white in colour, smaller and larger size and finally irregular and regular in shape indicates the presence of two different strains (Plate 2 and Table 4). All the two strains were in motile condition.

Table 4: Morphological characteristics of the selected bacterial isolates

	PSB - 8	PSB - 11
Colony size	Large	Small
Colour of colony	Creamish yellow	Creamish white
Surface	Regular	Irregular
Gram staining	+ ve Bacilli	- ve Bacilli
Motility	Motile	Motile

4.5. Biochemical characterization

The results of the biochemical characterization of the selected bacterial strains showed the presence and absence of tests results (Table 5). The 8 and 11bacterial strains exhibited similar positive results with catalase test, oxidase test, citrate utilization test, gelatin hydrolysis test, protease and ammonia test. All the two strains depicted negative results for indole test and urease test that showed no colour changes. Methyl red test and VogesProskauer test of PSB - 8 gave negative results. The positive result in catalase test showed bubbles formation, oxidase test indicated blue colour, methyl red test indicated ring formation, VogesProskauer test indicated ring formation, citrate test indicated colour separation, urease test showed pink colour, gelatinase test indicated semi-solid state, protease test indicated clear zone formation and ammonia production indicated brown to yellow colour changes. The major mechanism associated with the solubilization of insoluble phosphate is the production of organic acids, accompanied by acidification of the medium.[42]The carbohydrate test with two strains indicated that positive results for triple sugar ions, glucose, lactose, maltose, fructose and galactose. Ultimately PSB - 11 with sucrose test denoted negative result. The strains of PSB - 8 with amylase test produced negative results. The results of amylase test indicated zone formation, TSI test showed red colour, sucrose, glucose, maltose, fructose and galactose test showed yellow colour changes. The PSB - 8 and PSB - 11 bacterial strains were selected for further analysis based on their efficiency. Chen and Lie [43] reported the rhizospheric soil contains water soluble C compounds mainly as carbohydrates, organic acids and amino acids. It was well known that increasing the number of microorganisms associated with plant rhizosphere due to its carbon concentration.

Table 5: Biochemical characteristics and Carbohydrate utilization of the selected bacterial strains

Biochemical Tests	PSB - 8	PSB - 11
Catalase	+	+
Oxidase	+	+
Indole test	-	-

Methyl red	-	+
VogesProskauer	-	+
Citrate utilization	+	+
Urease	-	-
Gelatinase	+	+
Protease	+	+
Ammonia	+	+
Amylase	-	+
Triple sugar ion	+	+

Carbohydrates utilization tests	PSB - 8	PSB - 11
Sucrose	+	-
Glucose	+	+
Lactose	+	+
Maltose	+	+
Fructose	+	+
Galactose	+	+

'+' indicates positive and '-' indicates negative

4.6. Determination of pH range of the bacteria, optimum pH for the growth and temperature tolerance

The isolates showed a wide range of dependence for both temperature and pH. The optimum pH for growth of the organism was found to be pH 7 (Table 6) and the optimum temperature was found out to be 37° C (Table 7). This is in accordance with the results of Illmer and Schinner study.[44]

Table 6: The pH range of the selected organism and the optimum pH for the growth

Isolated strains	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9	pH 10
PSB - 8	-	+	+	++	+	-	-
PSB - 11	-	+	+	++	+	-	-

Note: '+' indicates growth; '++' indicates optimum condition; '-'indicates no growth

Table 7: The optimum temperature for the growth of the selected organism

Isolated strains	25 °C	30 °C	37 °C	40 °C
PSB - 8	+	+	++	-
PSB - 11	+	+	++	-

Note: '+' indicates response; '++' indicates optimum more response; '-'indicates no response

4.7. Quantitative estimation of P solubilizing potential of the bacterial isolates in broth culture

In the quantitative assay, the two bacterial isolates were tested by growing them in Pikovskaya's liquid medium supplemented with 0.2 % of tricalcium phosphate $Ca_3(PO_4)_2$ as the insoluble source. The bacterial cultures were withdrawn after the sixth, eighth and tenth day of incubation at 37 °C for estimation of soluble P in the broth by using ICP-OES (5100 Agilent Technologies) (Table 8). The amounts of P solubilized by the two isolates were varied in different day's intervals. The solubilization efficiency of the isolates increased as the incubation period increased (1.966 ± 0.0136 mg/l for PSB - 8; 2.263 ± 0.019 mg/l for PSB - 11). After ten days of incubation, the insoluble sources of tricalcium phosphate $Ca_3(PO_4)_2$ showed minimum solubilization potential of 1.696 mg/l by PSB - 8 and 1.846 mg/l by PSB -11. The PSB - 8 and PSB - 11 strains are statistically significant ($P<0.05$) different according to Duncan's Multiple Range Test.

Table 8: Quantitative phosphate solubilizing potential of the selected bacterial isolates in broth culture

Isolated strains	Days	Solubilizing potential
PSB – 8	6th	1.696 ± 0.009b
	8th	1.856 ± 0.013c
	10th	1.966 ± 0.0136a
$F=df_{(2,8)}$		543.527***
PSB – 11	6th	1.846 ± 0.013a
	8th	2.076 ± 0.029b
	10th	2.263 ± 0.019a

*$F=df_{(2,8)}$ 286.544***Means ± S.E., in a column followed by same superscript letter between values did not differ significantly at 5% level by Duncan's Multiple Range Test. ***Significant at 0.05 % level, ns - non significant*
.$^{a, b, c}$ indicates different days of solubilization in these columns so it is differentiated in letter based style

4.8. *Influence of P solubilizing organisms on pH of the growth medium*

The influence of growth of PSB isolates on pH of the medium was assessed at different intervals (sixth, eighth and tenth day after incubation) using a pH meter (Elico) (Table 9). All the culture filtrates showed a decline in pH when the incubation period was increased. In our study, after ten days of incubation with tricalcium phosphate $Ca_3(PO_4)_2$ as the insoluble source showed maximum decline in pH: 5.2 ± 0.057 for PSB - 8 and 3.233 ± 0.066 for PSB-11. The findings were correlated with the earlier study[45]. The PSB - 8 and PSB - 11 strains are statistically significant (P<0.05) different according to Duncan's Multiple Range Test.

Table 9: Influence of phosphate solubilizing bacteria on pH of the growth medium

Isolated Isolates	Days	pH of the growth medium
PSB – 8	6th	5.2 ± 0.057[b]
	8th	5.733 ± 0.033[ab]
	10th	6.466 ± 0.033[a]
F=$df_{(2,8)}$		245.851***
PSB – 11	6th	3.233 ± 0.066[b]
	8th	4.166 ± 0.033[b]
	10th	5.233 ± 0.033[a]
F=$df_{(2,8)}$		51.987**

*Means ± S.E., in a column followed by same superscript letter between values did not differ significantly at 5% level by Duncan's Multiple Range Test. **,***Significant at 0.05 % level, ns - non significant.*

4.9. *Production of indole-3-acetic acid by P solubilizing bacteria*

Phosphate forms an important metalloprotein which is responsible for the synthesis of tryptophan, which in turn acts as a precursor for the production of IAA. Efficiencies of the best isolates on the production of IAA in the presence and absence of $Ca_3(PO_4)_2$ in the Pikovskaya's liquid medium supplemented with 0.1 % of tryptophan were estimated (Table 10). The results revealed that all the isolates produced IAA in the medium supplemented with tryptophan, and that there was further enhancement in IAA production by the isolates due to the addition of $Ca_3(PO_4)_2$ source. This may be due to the induction of high P solubilizing efficiency of the isolates, which results in the stimulation of IAA synthesis. Among the three isolates, PSB - 8 was found to produce more IAA (17.066 mg/l) followed by PSB - 11 (10.166 mg/l) in the presence of P than in its absence. The values are statistically significant (P<0.05) different according to Duncan's Multiple Range Test. Similar to our results, Swain et al[46] also observed enhancement of IAA production in response to increased L-tryptophan concentration in the medium. The limited counts of PSB (Table 2) and less production of Indole-3 acetic acid (Table 10) may be attributed as among the reasons for the stunted growth of agricultural crops in the present study area, and this deserves further investigation by using PSB strains as bioinoculants to overcome the stunted growth.

Table 10: Production of indole-3-acetic acid by phosphate solubilizing bacteria

Isolated isolates	IAA (mg/l)
PSBT - 8	17.066 ± 0.233
PSB - Alone	6.75 ± 0.166
F=$df_{(2,8)}$	0.642*
PSBT - 11	10.166 ± 0.088
PSB - Alone	4.6333 ± 0.088
F=$df_{(2,8)}$	0.700*

*Means ± S.E., in a column followed by same superscript letter between values did not differ significantly at 5% level by Duncan's Multiple Range Test. *Significant at 0.05 % level, ns - non significant.*

4.10. *Molecular identification of isolates*

The two isolates (PSB - 8, PSB - 11) which showed highest P solubilization efficiency were identified using molecular marker 16S rRNA. The data obtained from the partial 16S rRNA sequencing of the two strains was in the range of PSB8-1056 bp and PSB11-626 bp long. The PCR amplified products were subjected to BLAST. The querysequence revealed that the two strains were *Bacillus megaterium* and *Pseudomonas aeruginosa* (Figure 2). Both the sequences are submitted in the NCBI and accession number of sequences were summarised in the Table 11. The species with high P solubilization efficiency has also been isolated from three different zones (*Saccharum officinarum, Coriandrum sativum* and nonrhizospheric soil samples). Phylogenetic trees of the isolated species were constructed by using neighbour-joining method (Figure 2). In phylogenetic trees, the organism *Bacillus megaterium* (MT156329) 16S ribosomal RNA gene was genetically very close to other three *Bacillus* strains. *Pseudomonas aeruginosa* (MT156330) gene was closely related to *Pseudomonas stutzeri.* Earlier investigations have focused on molecular studies on PSB from different environment crops.[47,48] In our study, the rhizospheric soil samples were rich in microorganisms, especially *Bacillus* species. These findings are similar with the studyof Jasuja *et al*[49].

Table – 11: Name, similarity and GenBank accession number of the selected bacterial isolates

Isolated strains code	Name of the isolated stains	GenBank accession number	Nucleotides length (bp)	16S rRNA identity (%)
PSB8	*Bacillus megaterium*	MT156329	1056	92.45 %
PSB11	*Pseudomonas aeruginosa*	MT156330	626	96.39 %

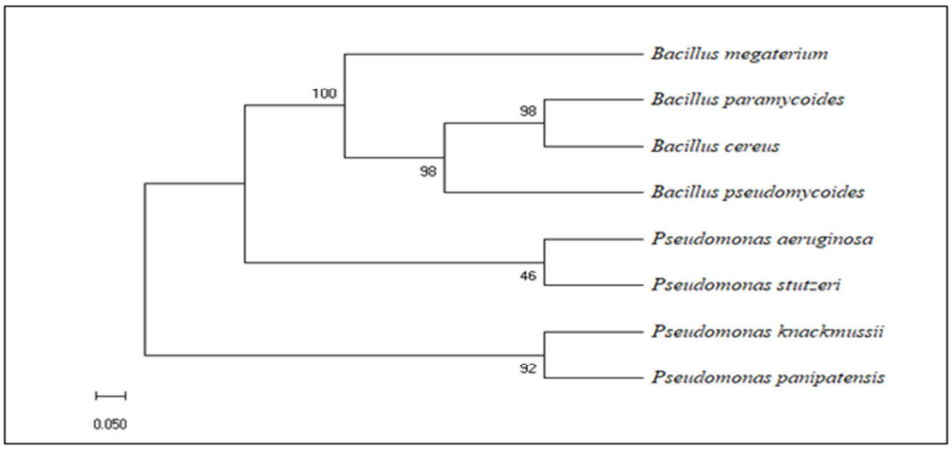


Fig 2: Neighbour - joining tree based on 16S rRNA sequences showing the phylogenetic relationship of two isolates by bootstrap method

To the best of our knowledge, this study has investigated the effect of phosphate solubilizing bacteria isolated from crop soils and their effect on sugarcane and coriander growth and P uptake. Phosphate solubilization activity of the most efficient PSB *Bacillus* sp. and *Pseudomonas* sp. were optimized for different environmental conditions for large-scale inoculum production. This strain with plant growth promoting attributes utilized different sugars and proved significant production of gluconic acid. This bacterium also increased the yield as compared to non- crop field along with P uptake and P release in soil.

5. CONCLUSIONS

Phosphorus is the least mobile element in plant and soil contrary to other macronutrients. Phosphate solubilization is a complex phenomenon, which depends on many factors such as nutritional, physiological and growth conditions of the culture. Identification of an elite strain capable of transforming unavailable forms of P into available forms will be an alternative tool to alleviate phosphate deficiency in plants. The above experimental study revealed that 2 strains that formed cleared zones in plate assay and available phosphate in broth assay were found to be potent to solubilize the phosphate and to produce plant growth regulator IAA. The plant growth promoting phosphate solubilizing bacterial strains are proved to be good alternatives of chemicals for increasing the plant growth, yield, help reduction in the use of hazardous agro-chemicals and used for bioinoculant. Although phosphorus PSMs are abundant in many of the soils, isolation, identification and selection of PSMs have not as yet been successfully commercialized, thus application is still found to be limited and also the large scale application of PSB in field level is still limited. Further studies are at progress to use the bacterial strains as bioinoculants for phosphate availability and growth stimulation to overcome the problem of stunted growth of agricultural crops.

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7. AUTHORS CONTRIBUTION STATEMENT

Dr. S. Sharmila, M.Anusha and S.Vinothini conceived of the presented idea. E.K.Ramya and S. Mownika verified the analytical methods. Dr.S.Sharmila to investigate and supervised the findings of this work. All authors discussed the results and contributed to the final manuscript.

8. CONFLICT OF INTEREST

Conflict of interest declared none.

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