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Biochemical profiling and Plant Growth –Promoting Abilities of isolated rhizosphereBacteria from Millet fields

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Abstract:

Both organic and inorganic forms of phosphorus are plentiful in soils; yet, they are typically a significant or even the main limiting factor for plant growth. Phosphate-solubilizing microorganisms (PSM) could play a significant role in providing phosphate to plants in a more sustainable and environmentally friendly way to avoid phosphorous deficit. Organic acids are created during the solubilization of phosphate. These organic acids function as chelators, which are substances that can move metals. While total phosphorous is present in soil in the form of organic compounds, the majority of them are inactive and unavailable to plants. Utilizing bacteria and inoculants that solubilize phosphorus concurrently increases crop production and plant uptake of phosphorus. From tricalcium phosphate, the isolated PSB released a significant amount of phosphorous. High amounts of phosphorous were released by the productive bacterial strains that were found in the rhizosphere soils. To avoid duplication among the isolates collected from the same sample, three isolates were chosen after eliminating isolates with comparable colony morphological traits. Tests on the PSB isolates' morphology and biochemistry and growth promotion study were conducted to identify them. These isolates were made available for molecular research. The strains were recognized as belonging to be members of *Burkholderiasp.*by 16s r RNA sequence analysis.

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Introduction:

Numerous bacterial species can influence plant growth favorably. Typically, these bacteria are found in the rhizosphere of plants and have this ability. A type of beneficial bacteria known as phosphate solubilizing microorganisms (PSM) is capable of hydrolyzing both organic and inorganic phosphorus molecules into soluble compounds. Notable PSMs are from Arbuscular mycorrhizal (AM), Actinomycetes, and bacterial (Bacillus, Pseudomonas, and Rhizobium) and fungal (Penicillium, and Aspergillus) species. Because the rhizosphere of several plants is where PSMs are known to be more metabolically active, PSMs were isolated from this area.

Plant growth is stimulated by a multitude of bacterial species, mostly those associated with plant rhizospheres. As a result, their application in agriculture as biofertilizers or control agents. This group of bacteria is known as plant growth-promoting rhizobacteria (PGPR). Several nations and businesses have utilized bacterial inoculants to boost plant yields. *Azotobacter*, *Rhizobium*, *Azospirillium*, and *Burkholderia* strains are typically used. The application of phosphorus fertilizers is the primary source of inorganic phosphate in agricultural soil. According to Walpola and Yoon (2012), cations fix roughly 70 to 90 percent of the phosphorus fertilizers supplied to the soil and transform inorganic phosphate.

The rhizosphere is home to a vast array of microorganisms, including the PGPRs. Plant development is directly impacted by PGPR through the production of phytohormones, the solubilization of inorganic phosphates, increased iron feeding because of siderophores that chelate iron, and volatile compounds that affect plant signalling pathways. Plant growth is promoted by a number of factors, including competition for nutrients and space, the development of systemic resistance in plants

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against a range of foliar and root diseases, antibiotics, and other harmful bacteria in the rhizosphere. The mineralization of organic phosphate by acid phosphatases and the production of organic acids by microorganisms, which lower soil pH, are the main processes in soil that lead to the solubilization of mineral phosphate.

Using phosphorus-solubilizing bacteria as inoculants increases phosphorus uptake. Phosphate solubilizing bacteria (PSB) can be applied singly or in combination to examine the impact on crop development and biomass output. This is because such microbial inoculants have the potential to significantly reduce the need for chemical fertiliser. One area of study is screening potential phosphate solubilizing isolates that can be used as bio-inoculants to boost plant development and yield. P is one of the essential nutrients that plants need to grow and develop. It contributes to a number of vital plant functions, such as respiration, energy metabolism, photosynthesis, nitrogen transport throughout the plant, and DNA transmission from one generation to the next. Thus, P is essential for tissue growth and cell division.In order to improve the energy balance of the system and decrease fertiliser consumption, the rhizosphere plays an increasingly important role in sustainable agricultural production. Rhizosphere activity facilitates the transformation, mobilisation, and solubilization of nutrients from a limited pool in the soil, allowing plants to absorb essential nutrients and realise their genetic potential for yielding crops. Microorganisms known as Plant Growth Promoting Rhizobacteria (PSB) interact with plants in the rhizosphere in a number of ways that are advantageous to plant growth and development, whether directly or indirectly. Kloepper and Schroth (1981).

MATERIALS AND METHODS

Sample Collection and isolation

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In Tamil Nadu, soil samples were taken in millet-growing regions such as Viliupuram, Srivilliputhur, and Edappadychose eight millet fields. The rhizosphere of millet plants is where the soil samples were taken. Samples were taken at 15 cm-deep sites. Polythene bags were used to collect the samples. After that, samples were collected for the study.

Screening of Phosphate Solubilizer

The isolates were picked with care after growing on Pikovskaya's medium containing tricalcium phosphate as a source of phosphorus (P). The bacteria that solubilized phosphate were isolated using the serial dilution and plating technique. Alok Ranjan (2013) states that after diluting soil samples to a 10^{-6} concentration, one mL of each 10^{-4}, 10^{-5}, and 10^{-6} concentration was added to a plate that contained sterilized Pikovskaya's agar media. The plates were incubated aerobically at 37^{0} degrees for nine to ten days. For further examination, the colonies with a clearly defined solubilization zone were chosen.

To purify the Phosphate Solubilizing Bacteria (PSB) obtained through the serial dilution and plating process, single colonies developed on particular media, such as Pikovskaya's agar, were selected.

Plate Assay for phosphate Solubilization by PSB Isolates

The isolates' capacity to disintegrate the insoluble tricalcium phosphate found in Pikovskaya's agar medium was assessed (Pikovskaya, 1948; Gupta et al., 1994). At each quadrant of Pikovskaya's agar plates, a loop full of culture cultured in the broth was spot-inoculated (3 mm thickness). Thus, PSB bacteria were cultured at 28±2 o C for five days, and their P solubilization was examined and compared in one plate with three replications. The bacterial colonies were surrounded by a distinct zone of phosphate solubilization, whose diameter was measured in millimetres.

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The following formula was used to get the PSB's phosphate solubilizing index (PSI):

Colony diameter plus Halazone diameter equals the phosphate solubilizing index.

Colony Width

Phosphate Solubilization by PSB Isolates using a Broth AssayThe Clescerie et al. (1998) method was utilised to quantitatively evaluate the phosphate solubilizing capability of PSB isolates.Following a 24-hour incubation period, 5 mL of sterile Pikovskaya's broth was added to the bacterial cultures, which were then shaker-agitated at 100 rpm for 10 days at 28 ±2 o C. Five millilitres of supernatant were collected in a screw-capped vial following centrifugation. The supernatant was then treated with 5 mLVanadomolybdate solution. The volume was made up to 25 mL and incubated overnight for yellow color development which was measured in each treatment, two replications were maintained. The phosphate solubilization per 5 g of tricalcium phosphate amended in one liter of broth was calculated using the standard phosphorous curve and expressed in mg L^{-1}.

PSB Isolates Morphology and biochemistry

Each bacterial isolate's pure culture was streaked in a loop on a specific agar medium using the quadrant streak method, and the colony pattern on thin agar plates was studied after 24-36 hours at 37°C. On agar plates, the colony morphology was inspected and recorded, including colony color, form, texture, margin, height, and transparency. The other morphological traits of bacterial isolates, such as cell shape, motility, Gram staining, and capsule staining, were also investigated.

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The bacterial species from distinctive colonies on Pikovskaya's Agar Media. Gram staining with (K00-1, Hi Media) was used to analyze the isolates' morphology by accepted practices. (Alok Rajan 2013) Under a compound microscope, the stained cells were examined.

Biochemical test

Oxidase Test, Nitrate Reductase Test, Catalase Activity, Indole Production, Methyl Red and Voges Proskauer Test, Citrate Utilization Test, Starch Hydrolysis, Cellulose Degradation.

Estimation of PGP Activity of the Isolates

Quantitative Estimation of Indole Acetic Acid (IAA)

IAA was quantified in accordance with the standard protocol outlined by Gordon and Weber (1951).

The isolates were incubated at 37 °C for an entire night after being infused with 5 millilitres of nutritional broth. After preparing 2 mg ml-1 stock in warm water, around 20µl aliquots of the culture broth were transferred into another 5 ml nutrient broth supplemented with L-tryptophan at a final concentration of 500µg ml-1. Following a 72-hour incubation period, the culture broth was centrifuged for 10 minutes at 3500 rpm to extract the supernatant. Vibrantly mixing one millilitre of the supernatant with four millilitres of Salkowski's reagent in a vortex mixer, the mixture was allowed to sit at room temperature for twenty minutes. The absorbance at 535 nm was then measured using a UV-visible spectrophotometer. The blank was a medium containing tryptophan that had not been infected and had been combined with Salkowski's reagent. The standard IAA curve was used to compare the IAA concentration in each culture medium.

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Gibberellic Acid Quantitative Estimation (GA)Gibberellic acid was quantitatively analysed using the standard protocol outlined by Holbrook et al. (1961).The 24-hour-old bacterial cultures were cultured for 48–72 hours at 28±2 o C after being infected with a particular broth. Every isolation has two replications kept up to date. Following incubation, 20 mL of the material was taken and centrifuged for 20 minutes at 4 o C at 4500 rpm. Two millilitres of zinc acetate solution were added to fifteen millilitres of the obtained supernatant, which had been transferred to another centrifuge tube. Two millilitres of potassium ferrocyanide solution were added at intervals of two minutes. After that, this mixture was centrifuged for 15 minutes at 4 o C at 2000 rpm. Following centrifugation, 5 mL of the supernatant was collected and combined with 5 mL of 30% HCL in a 15 mL glass container. After standing for 75 minutes at 20 o ±1 o C in a water bath, the absorbance value of this solution was measured at 254 nm. The gibberellic acid quality of the sample was assessed by consulting the standard curve that was created using chemical-grade gibberellic acid.

Calculating the Quantitative Production of Extracellular AmmoniaUsing a spectrophotometric approach, the excretion of extracellular ammonia was measured (Cappuccino and Sherman, 1999).Ten millilitres of peptone broth were used to inoculate 24-hour-old bacterial isolates, which were then cultured for 72 hours at 28±2°C. There were kept two replications. The cultures were centrifuged for 15 minutes at 4500 rpm following incubation. When Nessler's reagent was added, the supernatant began to take on a brown hue, indicating the presence of ammonia. This was interpreted as a favourable response for the synthesis of ammonia, as stated by (Cappuccino and Sherman, 1999). The amount of ammonia generated was calculated

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by measuring the absorbance of the colour created at 450 nm and comparing it to the ammonium sulphate reference curve.

Siderophore generationBy mixing two solutions, the modified Chrome Azurol Sulfonate (CAS)agar plate—described by Milagres et al., 1999—was used to qualitatively evaluate the siderophore production. After being autoclaved separately, the two solutions were gradually combined. After achieving a final volume of 100 millilitres, the solution was combined with 900 millilitres of pH 7 succinate agar medium, then transferred into sterile Petri dishes and left to harden. Bacterial isolates that were 24 hours old were spot-injected and allowed to incubate for 24 to 72 hours at 28±2 o C. The presence of siderophore was revealed by the colouring zone.

Solubilization of potassiumUsing an Aleksandrov medium, the potassium solubilization potential of a subset of bacterial isolates was evaluated in accordance with the methodology outlined by Lu and Huang (2010). After potassium aluminosilicate was synthesised in an insoluble state, autoclaving was used to sterilise the Aleksandrov medium. After melting and cooling to 45°C, this was poured to a 3 mm thickness into Petri plates that had been sterilised. Bacterial isolates were spot-injected onto the plates, and they were then cultured at 28±2oC for 7 days. It was noted where potassium solubilization occurred. Three replication plates were kept for every isolate.

Solubilization of silicatesThe plate assay method, as reported by Vasanthi et al. (2013), was used to evaluate the bacterial isolates' capacity for silicon solubilization. Using Bunt and Rovira agar medium supplemented with 0.25 percent magnesium

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trisilicate, agar plates with a thickness of 3 mm were created. Spot inoculation in the event that three replications of the bacterial isolates were maintained. After incubating the plates for roughly seven days, a distinct zone of solubilization was looked for.

Molecular characterization

The selected isolates were further subjected to molecular characterization by the agarose gel electrophoresis of the PCR amplified product showed bands of approximately 1.5 kbp size (Plate no.12). The results obtained by 16s rRNA gene sequencing are presented in (Table 16) and query cover, percent identity, best match in GenBank database of the isolates is given (Table 17).

Results and Discussion

Sample Collection and isolation

Twenty five bacterial isolates capable of phosphorous solubilizing bacteria were obtained from the collected soil samples. The isolates were named as PSB1 to PSB 20. Only three superior isolates were selected based on the phosphorous solubilization. The isolates were given code numbers from PSB 2, PSB 9, and PSB13.

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Screening of Phosphorous solubilizing bacteria by use of Pikovskayas agar

Tricalcium phosphate can be hydrolyzed by phosphatase enzymes produced by phosphorus solubilizing bacteria, which can then be used as an energy source. The phosphatase enzyme that produces the bacterial colonies exhibited a hydrolytic effect on the Pikovskayas agar medium, resulting in a distinct, whitish zone that surrounded the colonies. The diameter of the clear zone represents the enzyme produced by phosphorous-solubilizing bacteria's capacity to solubilize phosphorus. As a selective medium, pikovskayasagar was employed to promote the growth of bacteria that are able to use tricalcium phosphate. Since phosphate-solubilizing microbes can readily solubilize it, it can be utilised to detect P solubilization by bacterial isolates with effectiveness (Bashan et al., 2013). Pikovskayas agar is used to screen and isolate phosphate-solubilizing bacteria and specific bacterial colonies with measured widths of clear zone based on phosphate solubilization. Phosphatase activity was identified in the current study by selecting bacterial colonies that formed within a given incubation period and had an appropriate clear zone diameter. PSB1, PSB2, PSB3, PSB4 PSB5, PSB6 PSB7, PSB8, PSB10, PSB11, PSB12, PSB13, PSB14, PSB15, PSB16, PSB17, PSB18, PSB19, and PSB20 were the labels assigned to the isolates. Only three isolates with a clearance zone diameter greater than 10 mm were deemed noteworthy

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based on phosphate solubilization. To facilitate more investigation, the isolates were subcultured independently.

Plate assay for Phosphate solubilization by PSB isolates

The isolation of phosphate solubilizing bacteria was reported in cereals and millet crops using insoluble phosphate amended medium and many were isolated and selected based on the zone of clearance shown by the potential P solubilizing bacteria (Kour *et al.*, 2020a; Kour *et al.*, 2020b; Tahir *et al.*, 2013; Suleman *et al.*, 2018). Like this in our current study, three (PSB1, PSB2, PSB3) isolates were selected based on the phosphate Solubilization. The diameter of the clear zone, colony diameter, and hydrolytic values of selected isolates are indicated in Table 1. The highest hydrolytic zone was seen in PSB13 and the lowest value was seen in PSB9 and PSB2.

Table 1. Total number of PSB isolates from rhizosphere soils

NO	Name of the isolates	Zone of diameter
1	PSB1	0.5
2	PSB2	1.5
3	PSB3	0.2

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4	PSB4	0.3
5	PSB5	0.1
6	PSB6	0.2
7	PSB7	0.1
8	PSB8	0.3
9	PSB9	2
10	PSB10	0.2
11	PSB11	0.4
12	PSB12	0.3
13	PSB13	2.3
14	PSB14	0.5
15	PSB15	0.4
16	PSB16	0.5
17	PSB17	0.4
18	PSB18	0.5
19	PSB19	0.2
20	PSB20	0.2

Morphological Characters of the Selected Isolates

Morphological and microscopic characteristics of the PSB isolates are presented in Table 2 and Table 3. The colony characters such as colony shape, colony size, colony color, elevation, margin, opacity, and texture were visually noted. The majority of the isolates had raised round-shapedmucoid colonies with yellow in color and entire margin. The colony size range was 1mm. The colonies of PSB2, PSB 9, and PSB13 were yellow in color with the entire margin. The isolates were rounded in shape. Gram staining of the cells revealed that three isolates were Gram-negative and rod in shape.

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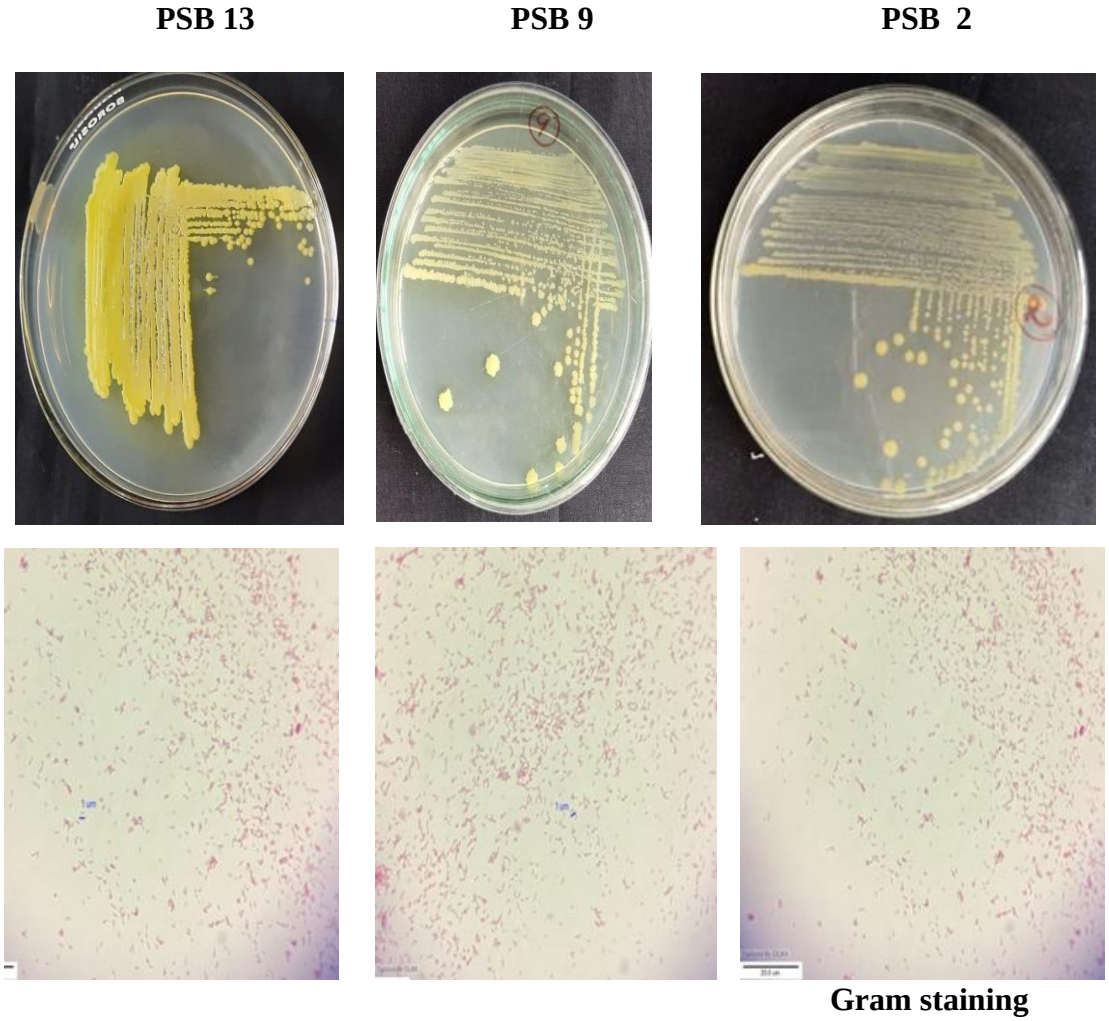

Gram staining

Table 2 Colony characteristics of the PSB isolates.

Sl. No.	Isolates	Colony Shape	Colony Margin	Elevation	Colony Colour	Opacity	Texture
1	PSB 2	Round	Entire	Raised	Yellow	Translucent	Mucoid
2	PSB 9	Round	Entire	Raised	Yellow	Translucent	Mucoid
3	PSB 13	Round	Entire	Raised	Yellow	Translucent	Mucoid

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Table 3. Morphology and microscopic characteristics of the PSB isolates.

Sl. No.	Isolates	Cell Shape	Arrangement	Motility	Gram staining
1	PSB 2	Rod	Single rod	Non motile	Negative
2	PSB 9	Rod	Single rod	Non motile	Negative
3	PSB 13	Rod	Single rod	Non motile	Negative

Biochemical characters of selected isolates

Various biochemical tests were carried out for all three isolates. Most of the isolates showed negative reactions for the indole production test. Voges Proskauer test, Glucose, Citrate utilization test, and cellulose degradation were also negative for all isolates. Also, the isolate showed a negative reaction towards the test, for Catalase, Arabinose, lactose, Sorbitol, Mannitol, Rhamnose, Sucrose, and Fructose also had positive reactions except for PSB2. The reactions like the Nitrate reduction Urease test and oxidase test are also positive reactions for all the isolates. Starch hydrolysis and cellulose degradation were also negative for all the isolates. Table 4.

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Biochemical Test

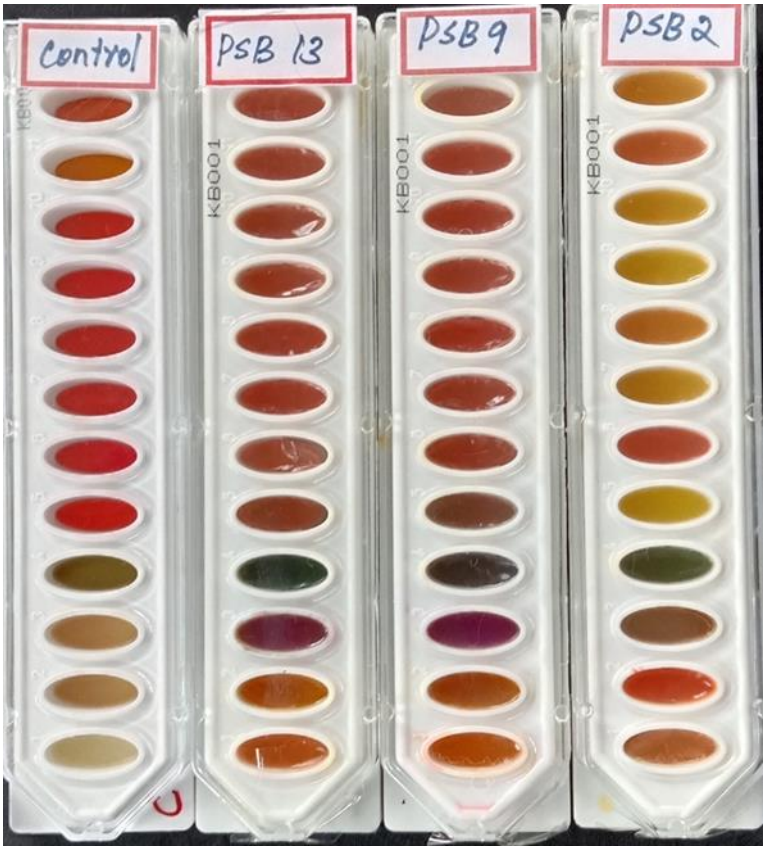

Table 4 Biochemical characteristics of the PSB isolates.

No	Test	PSB2	PSB9	PSB13
1.	Indole	-	-	-
2.	Methyl Red	+	+	+
3.	VoguesProskour	-	-	-
4.	Starch hydrolysis	-	-	-
5.	Cellulose Degradation	+	-	-

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6.	Lactose	+	-	-
7.	Sorbitol	+	-	-
8.	Mannitol	+	-	-
9.	Rhamnose	-	-	-
10.	Sucrose	+	-	-
11.	Arabinose	+	-	-
12.	Oxidase	+	+	+
13.	Urease	-	+	+
14.	Nitrate Reductase	-	+	+
15.	Glucose	-	-	-
16.	Fructose	+	-	-
17.	Catalase	+	+	+

Molecular Characterization

16srRNA analysis and molecular characterization potential PSB bacteria.

For the purpose of identifying the isolates at the generic level, 16 S rDNA sequencing was carried out for the promising PSB isolates PSB 2, PSB9, and PSB13. The gene sequence was then compared with the information currently available in GEN Bank using BLAST homology search. PSB 2 and PSB 9 showed strong similarities to Curtobactrium citrum, PSB 13 to Burkholderiacepacia, and PSB 9 to Burkholderia seminalis.

PGP activity of the isolates

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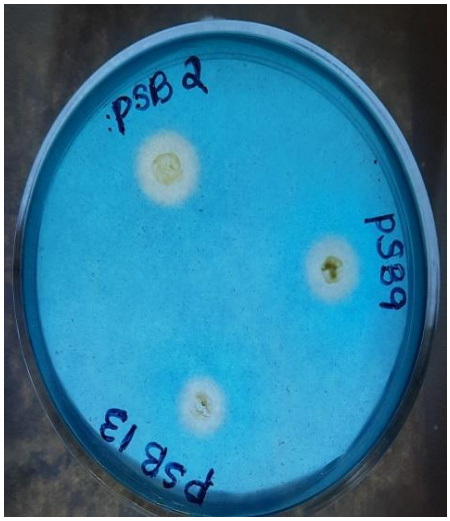
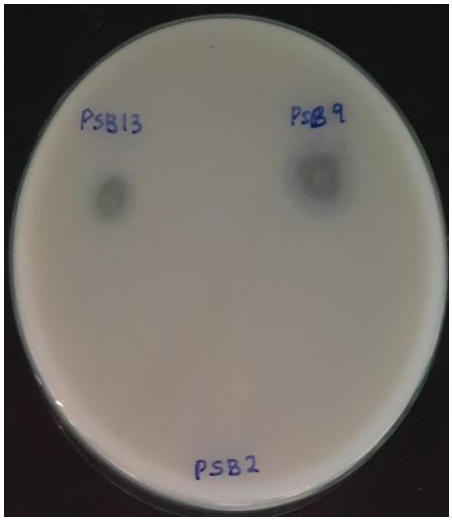
In the present study, all the 3 isolates were subjected to analysis of plant growth promotion activity such as IAA,GA, and ammonia production under invitro conditions considering the advantages of such screening techniques. Secondary metabolites such as natural phytohormones production by bacterial isolates promote plant growth (Restu*et al*., 2019).



Siderophore production



Potassium solubilization

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Table 5 Estimation of PGP activity of the isolates

S.No	Activity	PSB2	PSB9	PSB13
1	IAA (μgmL^{-1})	18.74	29.88	75.31
2	GA (μgmL^{-1})	81	62.68	75.05
3	Ammonia Productions ($\mu molmL^{-1}$)	10.81	3.52	3.30
4	HCN Production	Negative	Negative	Negative
5	Siderophore Production	Positive	Positive	Positive
6	ACC deaminase activity	Positive	Negative	Negative
7	Silicate solubilization	Negative	Positive	positive
8	Potassium solubilization	Negative	Positive	Positive
9	Zinc solubilization	Negative	Negative	Negative

The quantitative estimation of IAA production by the bacterial isolates can be assessed using the L-tryptophan amended specific broth. IAA is the most important

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toauxinsynthesis using tryptophan as the precursor. It promotes plant cell division, root elongation,etc.(Fett et al 1987,Raheem et al.,2018)In this study highest IAA production was reported by PSB 13 (75.31 µg mL-1) followed by PSB 9(29.88µg mL-1) and PSB 2 (18.74 µg mL-1).

Gibberellins act by modifying plant morphology and are involved in the elongation of plant tissue, particularly the stem, also Induce the plant uptake of minerals, Sugars, and Proteins.(Atzom*et al* 1988).Gibberellic acid plays an important role in plants switching from the vegetative stage to the reproductive stage. It also aids in seed germination, flowering, and fruit production in plants (Dong et al., 2017; Ma *et al.*, 2018).

All three of the chosen isolates were subjected to quantitative determination of gibberellic acid, with strain PSB 13 producing the highest amount of GA (75.05 µg mL-1), followed by strains PSB 9 (62.68 µg mL-1) and PSB 2 (81.09 µg mL-1).

By generating ammonia, which builds up and gives host plants nitrogen, the bacteria that promote plant development helped the plants they were growing. This helped plants by increasing biomass and root and shoot elongation in 78 different ways (Singh et al., 2019). Ensiferadhaerensstrain TMX-23 was reported to produce ammonia, which was confirmed by the addition of Nessler's reagent. The bacterial isolates PSB 9 (3.52µmol mL−1), PSB 13 (3.30µmol mL−1), and PSB 2 (10.81 µmol mL−1) recorded the highest amount of extracellular ammonia production on quantification with Nessler's reagent. (Zhou et al., 2013). According to Sandhya et al. (2017), Pseudomonas spp., Sinorhizobium sp., and Enterobacter sp. were among the plant growth-promoting endophytes from maize that were also engaged in the synthesis of ammonia.

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Siderophores are low-molecular-weight substances that, in situations where iron is scarce, chelate the ferric ion (Fe 3+) with high specific activity and serve as carriers of Fe (III) into microbial cells. By spot-inoculating the isolates onto chrome azurol S (CAS) agar plates, the existence of siderophore production was evaluated. The findings indicated that, of the bacterial isolates, only Aeromonas hydrophiliain all three isolates produced siderophore. Gupta and Gopal (2008) investigated the capacity of bacteria that promote plant growth to produce siderophores. Their findings showed that, in the presence of iron limitation, Pseudomonas fluorescens, Azospirillumbrasilense, and Enterobacter sp. were able to produce siderophores.

While PSB2 did not exhibit potassium solubilization, the chosen isolates PSB9 and PSB13 did so in medium containing potassium.By creating a distinct zone of solubilization around the bacterial colonies on agar plates, none of the chosen isolates were able to dissolve zinc present in the media or silicates contained in the media in the form of magnesium trisilicate.

Acidification is the general mechanism by which silicates are soluble. The absence of certain organic acids (citric, malic, tartaric, and gluconic acid), which aid in the solubilization of magnesium trisilicate, may be the cause of the lack of silicon solubilizing capacity. According to Vasanthi et al.'s (2016) study, the degree of solubilization varied depending on the species, kind of silicate mineral utilised, and length of incubation time. Bacterial isolates that did not solubilize silicate in plate test were nevertheless able to do so in broth assay. In their investigation, they discovered that distinct Burkholderiaspp. produce a range of organic acids, such as citric, tartaric, malic, hydroxy propionic acid, gluconic, and oxalic acid. According to the current study, PSB2, PSB 9, and PSB 13 may be potential phosphorous-solubilizing bacteria that have the ability to promote plant growth. The increased generation of GA and

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IAA by these particular isolates may be the cause of the increase in plant growth promotion. This study has revealed the potential for Curtobacterium citrum (PSB2), Burkholderia seminalis (PSB9), and Burkholderiacepacia (PSB9) to be used as biofertilizers for sustainable agriculture. These three bacteria are observed to be effective phosphate solubilizers with high plant growth boosters. After more analysis, they can be used for commercial production.

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