Isolation and characterization of phosphorus solubilizing bacteria, potassium releasing bacteria and sulphur oxidizing bacteria in maize (Zea mays L.) rhizosphere soils of Andhra Pradesh

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Abstract
In this study, the Phosphorus Solubilizing Bacteria (PSB), Potassium Releasing Bacteria (KRB) and Sulphur Oxidizing Bacteria (SOB) strains were acquired from maize rhizosphere soils from Guntur, Kurnool and Vizianagaram districts of Andhra Pradesh state. Those isolates were identified on different medias like, Pikovskaya’s agar, Aleksandrow’s agar and Thiosulphate agar medias followed by PSB, KRB and SOB isolates respectively. Those isolates were studied by their gram reaction, cultural, morphological, biochemical and PGPR characteristics.

Keywords: Rhizosphere, biochemical, PSB, KRB, SOB, PGPR

1. Introduction
The continuous use of inorganic fertilizers to buildup crop productivity and soil fertility after results in unexpected harmful environmental effects, including leaching of nitrate into ground water, surface runoff of phosphorus and nitrogen run-off, and eutrophication of aquatic ecosystems (Adesemoye and Kloepper, 2009) [1]. Bio-inoculants is a substance, which contains the living microorganisms that colonizes the rhizosphere and encourages production by increasing the supply of plant nutrients (Vessey, 2003) [2]. Bio-fertilizers promotes plant growth by fixing nitrogen, phytohormones production, phosphate solubilization and potassium releasing (Bashan et al., 2004) [3].

Phosphorus (P) is one of the essential macronutrients for biological growth and development. The simultaneous use of PSB as inoculants increases phosphorus intake resulted in enhanced plant growth and crop yield (Rodriguez and Fraga, 1999) [4]. Potassium (K) is essential in all cell metabolic processes and involves with photosynthesis, carbohydrate translocation, protein synthesis, disease resistance and drought tolerance. Consequently, K deficiency becomes a problem because K decreases easily in soils due to crop uptake, runoff, leaching and erosion (Sheng and Huang, 2002) [5]. Sulphur (S) requirement as it is an important component of plant proteins (amino acid synthesis) and chlorophyll formation. The Sulphur deficiency was characterized by a yellowing of the younger leaves followed by reddening of stems and leaves starting from leaf edges and gradually spreading to midrib and older leaves remains green (Rahul et al., 2017) [6].

The fertilization of the soil is very important to increased yield. However, commercial fertilizers are expensive means of supplementing essential nutrients to soil for plant growth. Inoculation of rhizobacteria increases plant growth parameters. Keeping this in view the present study is proposed to isolate and characterize the in vitro phosphate solubilising, potassium releasing and sulphur oxidizing bacteria from different soils and selected efficient isolates based on their PGPR characteristics.

2. Materials and Methods
2.1 Soil Samples Collection
The rhizosphere soil samples were collected from different districts, Guntur, Kurnool and Vizianagaram for the isolation of PSB, KRB and SOB bacterial isolates.
The soil samples were mainly collected from maize rhizosphere. Crop plants were selected randomly in the field and the intact root system was dug out, carefully taken in plastic bags, labeled well and stored at 4 °C.

2.2 Isolation Technique

2.2.1 Serial dilution technique

One gram sample of soil serially diluted up to 10⁻⁶ dilutions and 0.1 ml each of the aliquots from 10⁻², 10⁻³ and 10⁻⁴ dilutions was transferred to already solidified Pikovskaya’s agar, Aleksandrov’s agar and Thiouosphate agar medium. After plating with Pikovskaya’s agar, Aleksandrov’s agar and Thiouosphate agar medium, the plates were incubated in an inverted position for 3-5 days at 30 °C. Characteristic Solubilization zones around colonies growing over the medium in PSB, KRB isolates followed Pikovskaya’s agar, Aleksandrov’s agar medium but yellow colour colonies in SOB on Thiouosphate agar medium. Further, the PSB, KRB and SOB isolates were purified by the streak plate method and well isolated colonies on the plates were preserved on their respective agar slants. Isolates were maintained on slants at 4 °C in a refrigerator for further use.

2.3 Morphological Characterization

All the isolates were checked for their purity and then studied for the colony morphology and pigmentation. The cell shape and gram reaction were also recorded as per the standard procedures given by Barthalamew and Mittewar (1950) [2].

2.4 Colony Morphology

The morphological characteristics of the colony of each isolate were examined on Nutrient agar medium by incubating for specific period. Cultural characterization of isolates such as shape, size, elevation, surface, margin and colour of the colony were recorded.

2.5 Biochemical characterization of PSB, KRB and SOB isolates

2.5.1 Indole Production

Sterilized Hydrogen Sulfide-Indole-Motility agar (SIM agar) slants or Tryptophan broth tubes were inoculated with the overnight cultures of the isolates and incubated for 48 h at 28 ± 2 °C. Following incubation, 10 drops of Kovac’s indole reagent was added to each tube. The isolates showing the production of red color was recorded as positive for indole production (Cheesbrough, 2006) [7].

2.5.2 Methyl Red Test

Sterilized glucose-phosphate broth tubes were inoculated with the test culture and incubated at 28 ± 2 °C for 48 h. After incubation five drops of methyl red indicator was added to each tube and gently shaken. Red color production was taken as positive and yellow color production was taken as negative for the test (Olutiola et al., 2000) [11].

2.5.3 Voges Prusker’s Test

To the pre sterilized glucose-phosphate broth tubes, test cultures were inoculated and incubated at 37 °C for 48h. After incubation ten drops of Barritt’s reagent, A was added and gently shaken followed by addition of 10 drops of Barritt’s reagent B. Development of pink color in the broth was taken as positive for the test (MacFaddin, 2000) [10].

2.5.4 Citrate Utilization

Isolates were streaked on Simmon’s citrate agar slants and incubated at 28 ±2 °C for 24 h. Change in color from green to blue indicates the positive reaction for citrate utilization (Cappucino, 1983).

2.6 Screening of bacterial isolates for plant growth promoting traits

2.6.1 Siderophore Production

Siderophore production was estimated at qualitatively. Chrome azurol sulphonate (CAS) agar medium (Schwyn and Neilands, 1987) was used for the detection of siderophores, isolates were grown in synthetic medium, containing 0.5 µM of iron and incubated for 24 hrs on a rotary shaker at room temperature. CAS assay is used to detect the siderophores. The CAS plates were used to check the culture supernatant for the presence of siderophores. Culture supernatant was added to the wells made on the CAS agar and incubated at room temperature for 24 hrs. Formation of yellow to the orange colored zone around the wells indicates siderophore production.

2.6.2 In-vitro assay for phosphate solubilizing activity

Phosphate solubilization activity was determined using Pikovskaya’s agar medium containing 0.5% (W/V) Ca₃(PO₄)₂ (Pikovskaya, 1948) [15]. Pikovskayas agar plates were prepared and sterilized. The inoculums were spot inoculated on the pikovskayas plate. 24 hrs old culture was used for the inoculation. The plates were incubated for 72-96 hrs at room temperature. The clear zone was observed around the spotted area after the incubation period.

2.6.3 HCN Production

The HCN production was tested by the method of Castric and Castric (1983) [6]. First respective media plates i.e., Pikovskaya’s broth (PSB), modified Aleksandrov’s agar media (KRB), thiosulphate agar was prepared separately and incubated for 24 hrs. After that, 1 ml of culture of each test isolate was inoculated on respective media plates separately. A disc of Whatman filter paper no.l of the diameter equal to the petri plate size, impregnated with alkaline picric acid solution (0.5% picric acid (w/v) in 1% sodium carbonate) was placed in the upper lid of the inoculated petri plates under aseptic condition. The control plate did not receive the inoculum. The plates were incubated upside up at 28 °C±2 °C for 48-72 hrs. Change in colour from yellow to light brown, moderate or strong reddish brown was taken as indication of HCN production.

3. Results and Discussion

3.1 Collection of soil samples

The rhizosphere soil samples were drawn from Guntur, Kurnool and Vizianagaram districts of Andhra Pradesh, where maize is grown and geographical indications were recorded at sampling sites (Table 1).

3.2 Isolation of phosphorous solubilizing bacteria (PSB), potassium releasing bacteria (KRB) and sulphur oxidizing bacteria (SOB) from rhizosphere soil sample

The phosphorus solubilising bacteria (PSB), potassium releasing bacteria (KRB) and sulphur oxidizing bacteria (SOB) populations in the rhizosphere soils were determined in (Table 2 and plate 1) and coding of isolates according to district, mandal, village and isolate identified were shown in (table 3). PSB population ranges between 3.82-12.46 × 10⁶ CFU g⁻¹ soil. Maximum PSB population were recorded in the village Nelapadu, Guntur district (12.46 × 10⁶ CFU g⁻¹ soil) and least population was attained in the soils of Kolakaluru,
Guntur district (3.48 × 10^6 CFU g⁻¹ soil). Similar results were shown by Pandey and Chayanika (2018) who obtained 10 PSB isolates from the soil sample of rhizosphere on Pikovskaya’s agar medium.

The KRB population ranges between 2.42 - 9.80 × 10^6 CFU g⁻¹ soil. Maximum KRB population was recorded in soil sample from Bethapudi, Guntur district (9.80 × 10^6 CFU g⁻¹ soil) and minimum KRB population was obtained in soil sample of Singupalem, Guntur district (2.42 × 10^6 CFU g⁻¹ soil). Similar results was observed by Parmar et al. (2016) [13] isolated potassium solubilizers from rhizosphere soils, using Aleksandrow’s media.

SOB population ranges between 1.66 - 4.28 × 10^6 CFU g⁻¹ soil. Maximum SOB population was recorded in soil sample from Borabanda, Vizianagaram district (4.28 × 10^6 CFU g⁻¹ soil) and minimum SOB population was recorded at rhizosphere soils of Rajupeta, Vizianagaram district (1.34 × 10^6 CFU g⁻¹ soil). Similar results was observed by Reddy et al. (2018) [17] to isolated sulphur oxidising bacteria on thiosulphate agar media from saline soils of groundnut growing places in Andhra Pradesh, India.

### 3.3 Cultural and morphological traits of PSB, KRB and SOB isolates

After isolation of PSB, KRB and SOB isolates were studied for morphological and cultural traits (Table 4). Among the 4 PSB isolates were mostly irregular shaped, light yellow colour and rough colonies were observed. All PSB isolates was gram -ve reaction and rod shaped. Comparable results was reported by Paul and Sinha (2016) [14] the phosphate solubilising bacterium was identified using physiological, morphological characters and these isolates were showed gram -ve, rod shaped and motility.

The KRB isolates were mostly round shaped, creamy colour and smooth colonies were observed. All KRB isolates was gram +ve reaction and rod shaped. Similar results was reported by Kavya et al. (2020) [9] studied characteristics of six isolates of K releasing bacteria from different places of Andhra Pradesh. Majority of the isolates were identified as entire smooth margin, raised, translucent, gram +ve rods and whitish to creamy in appearance. Among all SOB isolates were mostly round shaped and creamy colour, smooth colonies, gram -ve reaction and rod shaped were observed. Similar results was observed by Veerender et al. (2014) [22] total of 16 bacterial isolates are drawn from different ecosystems. The screened isolates were resulted as Gram negative, rod shaped; smooth, concentric and white colour colonies.

### 3.4 Biochemical and physiological characterization

The study of phosphorus solubilising bacteria, potassium releasing bacteria and sulphur oxidizing bacteria were checked against biochemical tests viz., methyl red test, voges proskauer’s test, citrate utilization test and indole production (Table 5).

All four isolates of (PSB), 3 isolates are positive for citrate utilization and methyl red tests. All the isolates were positive for voges proskauer’s and indole production. From three of potassium releasing bacteria, one isolate (GRBK) showed negative and remaining positive for citrate utilization test. For voges proskauer’s, methyl red and Indole production all isolates showed positive results. Among all the isolates of Sulphur oxidizing bacteria (SOB) were showed positive results for voges proskauer’s and methyl red test. For Citrate utilization test isolate VBKS and indole production test isolate VSBS showed negative results and remaining all isolates were showed positive.

Similar results were observed by Bashir et al. (2018) [4] isolated PSBs from various soil samples of rhizosphere and performed various tests viz., citrate utilization test, indole production test and gas production. Saha et al. (2016) [14] characterized potassium releasing bacteria based upon their morphological and biochemical characteristics. Reddy et al. (2018) [17] to isolated sulphur oxidising bacteria on thiosulphate agar media and those isolates were characteristics based upon their biochemical characteristics.

### 3.5 Screening of bacterial strains for plant growth promoting (PGPR) properties

Among all isolates were screened by PGP activities viz., phosphorus solubilization, siderophore and HCN production (Table 6 and plate 2). Among all PSB isolates GTAP recorded the phosphate solubilization zone 6.2 mm diameter was heighest. For HCN isolates i.e., GTAP, GTCP, GTKP and siderophore production GTAP and GTCP isolates were showed positive results. The KRB isolate GRPK isolate was formed a clear zone of phosphate solubilization which ranged 4.1mm. For HCN production GRPK and GRSK isolates were shown positive results. For siderophore production GRPK isolate give positive result. Out of all SOB isolates, only one isolate VBRS were formed clear zone of phosphate solubilization zone and HCN production. For siderophore production VBRS and VSBS isolates give positive results.

Similar results were observed with Habibi et al. (2019) [8] were isolated 98 bacterial strains and their cultural, morphological and PGP traits, such as indole-3-acetic acid production, acetylene reduction, phosphate and potassium solubilization, and siderophore production were evaluated.

### Conclusion

The present study revealed that that isolation, cultural, morphological, biochemical and PGPR characterization of phosphorus solubilising, potassium releasing and sulphur oxidizing bacteria are not just necessary to understand their ecological function in the rhizosphere rather utilization in eco-friendly and improves the soil health, fertility conditions. Out of 10 isolates, for PSB (GTAP), KRB (GRPK) and SOB (VBRS) were selected as efficient isolates by their PGPR activities. These efficient isolates were used for further field application as microbial inoculants to enhance plant growth parameters and yield attributes.

| S.No | Latitude No | Longitude No | District | Mandal | Village          | Soil Type |
|------|-------------|--------------|----------|--------|------------------|-----------|
| 1    | 16° 24' 85.3'' N | 80° 62' 90.2'' E  | Guntur   | Tenali | Angalakuduru     | Black     |
| 2    | 16° 21' 53.5'' N | 80° 67' 22.1'' E  | Guntur   | Tenali | Chinnavaramu     | Black     |
| 3    | 16° 26' 44.2'' N | 80° 68' 54.7'' E  | Guntur   | Tenali | Nelapadu         | Black     |
| 4    | 16° 30' 00.4'' N | 80° 61' 69.9'' E  | Guntur   | Tenali | Kolalakuru       | Black     |
| 5    | 16° 29' 51.7'' N | 80° 65' 25.2'' E  | Guntur   | Tenali | Nandivelugu       | Black     |
| 6    | 16° 02' 34.0'' N | 80° 81' 42.9'' E  | Guntur   | Repalle | Bethapudi       | Black     |

~ 792 ~
### Table 2: Microbial population (CFU g⁻¹ soil) of PSB, KRB and SOB isolates

| S. No | Village       | Mandal | District | PSB population (10⁶ CFU g⁻¹ soil) | KRB population (10⁶ CFU g⁻¹ soil) | SOB population (10⁶ CFU g⁻¹ soil) |
|-------|---------------|--------|----------|-----------------------------------|-----------------------------------|-----------------------------------|
| 1     | Angalakuduru  | Tenali | Guntur   | 9.46                              | -                                 | -                                 |
| 2     | Chinna ravuru | Tenali | Guntur   | 8.51                              | -                                 | -                                 |
| 3     | Nelapadu      | Tenali | Guntur   | 12.46                             | -                                 | -                                 |
| 4     | Kolakaluru    | Tenali | Guntur   | 3.82                              | -                                 | -                                 |
| 5     | Bethapadi     | Repalle| Guntur   | -                                 | 9.80                              | -                                 |
| 6     | Singupalem    | Repalle| Guntur   | -                                 | 2.42                              | -                                 |
| 7     | Potumeraka    | Repalle| Guntur   | -                                 | 3.60                              | -                                 |
| 8     | Rajupeta      | Bobbili| Vizianagaram | -                             | -                                 | 1.66                             |
| 9     | Krishnapuram  | Bobbili| Vizianagaram | -                             | -                                 | 2.84                             |
| 10    | Borabanda     | Saluru | Vizianagaram | -                             | -                                 | 4.28                             |

### Table 3: The isolated bacterial codes according to the district, Mandal, village and isolate identified

| S.No | District | Mandal | Village       | PSB/KRB/SOB | Isolate code |
|------|----------|--------|---------------|-------------|--------------|
| 1    | Guntur   | Tenali | Angalakuduru  | PSB         | GTAP         |
| 2    | Guntur   | Tenali | Nelapadu      | PSB         | GTNP         |
| 3    | Guntur   | Tenali | Chinna ravuru | PSB         | GTCP         |
| 4    | Guntur   | Tenali | Kolakaluru    | PSB         | GTKP         |
| 5    | Guntur   | Repalle| Bethapadi     | KRB         | GRBK         |
| 6    | Guntur   | Repalle| Singupalem    | KRB         | GRSK         |
| 7    | Guntur   | Repalle| Potumeraka    | KRB         | GRPK         |
| 8    | Vizianagaram | Bobbili| Krishnapuram  | SOB         | VBKS         |
| 9    | Vizianagaram | Bobbili| Rajupeta      | SOB         | VBRS         |
| 10   | Vizianagaram | Saluru | Borabanda     | SOB         | VSBS         |

### Table 4: Morphological characteristics of PSB, KRB and SOB isolates

| S.No | Isolate name | Size     | Shape   | Cultural characters | Morphological characters | Gram reaction | Shape |
|------|--------------|----------|---------|---------------------|--------------------------|---------------|-------|
| 1    | GTAP         | Medium   | Round   | Light yellow        | Convex Smooth Entire     | Gram –ve      | Rod   |
| 2    | GTNP         | Small    | Irregular | Whitish Raised     | Rough Entire Gram –ve    | Rod           |       |
| 3    | GTCP         | Medium   | Irregular | Light yellow Convex| Rough Irregular Gram –ve| Rod           |       |
| 4    | GTPK         | Small    | Irregular | Light yellow Flat   | Smooth Irregular Gram +ve| Rod           |       |
| 5    | GRBK         | Medium   | Round    | Creamy Convex      | Smooth Entire Gram +ve   | Rod           |       |
| 6    | GRSK         | Small    | Round    | Creamy Convex      | Smooth Entire Gram +ve   | Rod           |       |
| 7    | GRPK         | Small    | Round    | Whitish Raised     | Smooth Entire Gram +ve   | Rod           |       |
| 8    | VBKS         | Small    | Round    | Creamy Smooth      | Regular Gram –ve         | Rod           |       |
| 9    | VBRS         | Medium   | Round    | White Convex       | Smooth Irregular Gram +ve| Rod           |       |
| 10   | VSBS         | Small    |Irregular | Creamy Convex      | Smooth Irregular Gram –ve| Rod           |       |

### Table 5: Biochemical characterization of PSB, KRB and SOB isolates

| S.No | Isolate name | Methyl red test | Voges -proskauer’s | Citrate utilization | Indole Production |
|------|--------------|----------------|--------------------|---------------------|-------------------|
| 1    | GTAP         | +              | +                  | +                   | +                 |
| 2    | GTNP         | +              | +                  | -                   | +                 |
| 3    | GTCP         | -              | +                  | +                   | +                 |
| 4    | GTKP         | +              | +                  | +                   | +                 |
### Table 6: PGPR characteristics of PSB, KRB and SOB isolates

| S.no | Isolate name | Phosphate solubilisation | Siderophore production | HCN production |
|------|--------------|--------------------------|------------------------|---------------|
|      |              | Zone diameter (mm)       | PSI                     |               |
|      |              | Solubilization zone      | Culture diameter        |               |
| 1    | GTAP         | 6.2                      | 4.8                    | 2.29          | +             | +             |
| 2    | GTNP         | 1.6                      | 2.5                    | 1.64          | -             | -             |
| 3    | GTCP         | 2.8                      | 6.1                    | 1.45          | +             | +             |
| 4    | GTKP         | 1.7                      | 3.8                    | 1.44          | -             | -             |
| 5    | GRBK         | -                        | 1.6                    | -             | -             | -             |
| 6    | GRSK         | -                        | 2.1                    | -             | -             | +             |
| 7    | GRPK         | 4.1                      | 4.3                    | 1.95          | +             | +             |
| 8    | VBKS         | -                        | 4.1                    | -             | -             | -             |
| 9    | VBRS         | 3.2                      | 3.4                    | 1.94          | +             | +             |
| 10   | VSBS         | -                        | 1.8                    | -             | +             | -             |

(+/Ve Positive) (-/Ve Negative)

**Plate 1:** Isolation of PSB, KRB and SOB (a, b and c) isolates

- **a)** Isolation of PSB
- **b)** Isolation of KRB
- **c)** Isolation of SOB

- **a)** P-Solubilization zone
- **b)** Siderophore production
c) HCN production

Plate 2: PGPR characteristics of PSB, KRB and SOB (a, b and c) isolates

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