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Isolation and Biochemical Characterization of Plant Growth Promoting Bacteria from a Maize Crop Field

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ABSTRACT

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Plant growth-promoting rhizobacteria (PGPR) are used as alternatives to the chemical fertilizers to increase crop yield in agriculture. The present study was undertaken to isolate, screen and evaluate selected promising PGPR isolates from maize fields. Out of 30 bacterial isolates, four most promising isolates (SP-01 to SP-04) have analysed for the various PGP traits. Bacterial isolate SP-03 showed the maximum PGP traits like N₂ fixation, IAA production, ammonia production and siderophore production in *in-vitro* condition. To identify the isolates morphological and biochemical tests were performed and analysed in the ABIS online biochemical identification system. These analysis confirmed isolates belonged to *Brevibacillus sp.* and *Panibacillus sp.* As per our knowledge, the present study one among the few studies of *Brevibacillus* and *Panibacillus sp.* which exhibit various PGP traits.

Introduction

Plant Growth Promoting Rhizobacteria (PGPR), which enhances plant growth and increase crop yield via secretion of various plant growth promoting substances as well as biofertilizers. PGPR's exhibit antagonistic effects to soil-borne pathogens or induce the systemic resistance against pathogens in the entire plant lifespan. A wide range of bacteria colonizes in the root as well as other parts of plant-like roots, stems, leaves, seeds, and fruits (Ryan *et al.*, 2018). The bacterial

community inhabiting, rhizosphere region perhaps source of formation of the community of endophytic bacteria in the plant (Hardoim *et al.*, 2008).

The rhizosphere region is a major hot spot of microbial interactions because of plant root exudates released by the plant acts as a food source for microorganisms and a driving force of their population density and activities (Klemetsson *et al.*, 1988; Berendsen *et al.*, 2012).

Antagonistic rhizobacteria produce several substances like siderophores and antibiotics, which indirectly promotes the growth in many plants by controlling plant pathogens. In plants pathogen-induced systemic acquired resistance (SAR) resembles with induced systemic resistance (ISR) when the inducing bacteria and the challenging pathogen remain spatially separated. Both types of induced resistance render uninfected plant parts more resistant to pathogens in several plant species. Rhizobacteria induce resistance through the salicylic acid-dependent SAR pathway or require jasmonic acid and ethylene perception from the plant for ISR (Beneduzi *et al.*, 2012).

Several bacterial spp. has been utilized as PGPR such as *Agrobacterium*, *Arthrobacter*, *Azotobacter*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Caulobacter*, *Chromobacterium*, *Erwinia*, *Flavobacterium*, *Micrococcus*, *Pseudomonas*, and *Serratia* (Bhattacharyya and Jha, 2012). Diversified *Bacillus* spp. Occurred in agricultural fields contribute to crop productivity by direct or indirect mechanisms. The major direct mechanisms of plant growth promotion include the production of phytohormones, phosphate solubilization and mobilization, siderophore production, antibiotics and induction of plant systemic resistance to pathogens (Kumar *et al.*, 2011). Indirect plant growth mechanisms include control of plant pathogens and deleterious rhizosphere inhabiting microbes (Karimi *et al.*, 2012; Xiang *et al.*, 2017).

In the present study, we collected a soil sample from maize field serially diluted in sterile water and 0.1 ml of aliquot spread on Nitrogen free Bromothymol blue (NFb) and Pikovskaya's medium and incubated at 30 °C. Later bacterial colonies were selected and subjected to evaluate the plant growth promoting properties.

Materials and Methods

Collection of soil sample

Soil samples were collected from rhizosphere (Maize) and Non-rhizosphere regions, from the agriculture land at the depth of 6 to 12 cm. Collected soil samples were stored in polythene bags aseptically.

Isolation of plant growth promoting bacteria

For isolation of plant growth promoting bacteria, 1g of rhizosphere and non-rhizosphere soil was suspended in 10ml of sterile water and then serially diluted and transferred to NFb and Pikovskaya's media by spread plate technique and incubated in an inverted position at 30 °C for 28-48 hrs. Isolated colonies appearing on agar plates were transferred to slants for further study.

Biochemical characterization of PGPR bacteria

Different biochemical parameters were performed to identify bacterial isolates. Preliminarily, Gram's staining technique, motility, growth at 5% NaCl along with Indole production test, Methyl red (MR) and Voges-Proskauer's (VP) test, Citrate utilization test, Catalase test, Starch hydrolysis test, Gelatine liquefaction test, Oxidase test, Nitrate Reduction test, and Carbohydrate fermentation tests were performed.

Plant growth promoting parameters of bacteria

Nitrogen fixation assay on NFb medium

Isolated bacteria were screened on NFb solid medium for nitrogen fixation activity. The composition of NFb (g/l): DL-Malic acid 5.0,

KOH 4.0, K₂HPO₄ 0.5, FeSO₄·7H₂O 0.05, MnSO₄·H₂O 0.01, MgSO₄·7H₂O 0.1, NaCl 0.02, CaCl₂·2H₂O 0.01, Na₂MoO₄·2H₂O 0.002, Bromothymol blue 2ml (0.5% alcoholic solution), Agar-agar 15, Distilled water 1000 ml, and pH 6.8). Isolates showed blue colour zone around the colony considered as positive for nitrogen fixation (Swamy *et al.*, 2016).

Phosphate solubilization

On phosphate solubilization activity the bacterial isolates were inoculated on Pi solid medium and incubated (30°C/48 hr). Further, the zone of clearance around the colony was observed for phosphate solubilization (Nautiyal *et al.*, 2000).

Ammonia production

Ammonia production was carried out according to Cappuccino and Sherman (1992). The bacterial isolates inoculated to peptone water and incubated at 30 °C/48 hr.

After incubation, 1ml of Nessler's reagent was added to each vials and ammonia production has qualitatively detected by colour development.

Indoleacetic acid (IAA) assay

3-Indoleacetic acid (IAA) assay has conducted in Tryptone yeast extract broth with tryptophan. Bacterial isolates were inoculated and incubated at 30°C/48 hr. The bacterial culture was centrifuged (10,000 g for ~20 min) and the supernatant was collected then performed the Salkowski method.

The pink colour production has visually observed and measured the colour intensity at 540 nm in the spectrophotometer (Swamy *et al.*, 2016).

Siderophore production assay

The isolates were screened for siderophore production by the universal chemical assay (chrome azurol S assay). Glasswares used in this study were washed in 6M HCl to remove traces of contaminating iron and then rinsed thoroughly with distilled water. Autoclaved CAS-HDTMA and MM9 media were plated and incubated for 24 hr at 30 °C for the detection of contamination. Later, the isolates were spot inoculated to CAS agar plates and incubated at 30 °C for 72 hr. The isolates producing orange colour with of halo zone around the colonies were considered as siderophore producers (Milagres *et al.*, 1999; Pérez-Miranda *et al.*, 2007; Ahmad *et al.*, 2008).

Results and Discussion

Isolation of plant growth promoting bacteria

A total of 30 bacterial colonies were isolated on both NFb and Pikovskaya's agar medium from the maize crop field. Out of 30 isolates 4 potential isolates i.e SP-01, SP-02, SP-03 and SP-04 showed PGPR activities were selected for the further studies. Soil contains a wide range of PGP bacteria helpful to plants in many ways and several previous studies employed serial dilution method to isolate the PGPR from soil samples (Gettha *et al.*, 2014; Verma and shahi 2015).

Biochemical characterization of PGPR bacteria

Biochemical tests were done for the isolates showed PGP traits and the results were tabulated (Table 1). Based on the morphological and biochemical analysis, isolates were confirmed as *Brevibacillus* sp. The rhizosphere bacterial isolates were characterized by biochemical attributes and

were identified as SP-01 (*Brevibacillus brevis* ~97.2% similarity), SP-02 (*Brevibacillus brevis* ~97.2% similarity), SP-03 (*Brevibacillus brevis* ~96.9% similarity) and SP-04 (*Bacillus aeolius* ~85% similarity) on the basis of ABIS online software (Table 1). *Bacillus* and *Paenibacillus* strains plant growth promoting traits have been widely studied for enhancement of plant growth (Choudhary and Johri 2008; Dev *et al.*, 2016).

Plant growth promoting parameters of bacteria

The four potential isolates were screened for their ability to fix nitrogen on the NFb solid medium in which SP-03 isolate gave a maximum zone of colour than other isolates (SP-01, 02 and 04 who had also shown nitrogen fixation activity (Figure 1 and 2). The nitrogen fixation was assessed by acetylene reduction assay showed a variation which ranged from 3.57 to 9.25 $\mu\text{mol C}_2\text{H}_4$ formed/mg protein/h and also analysed their plant growth promoting characters (Damodara chari *et al.*, 2015).

Phosphate solubilizing activity was estimated by point inoculating of bacteria. Out of 30 bacterial isolates, only SP-02 showed maximum activity with 30 mm in diameter on Pikovskaya’s agar medium (Figure 3). The maximum phosphate solubilization was recorded from the *Bacillus* and *Pseudomonas* sp. from the saline soil (Lamizadeh *et al.*, 2016).

The qualitative determination of ammonia production was recorded from the all 04 bacterial isolates, however, SP-02 showed the brown colour indicates maximum ammonia production.

All 30 bacterial isolates were subjected to IAA production and IAA was detected in the supernatant of ten isolates (Figure 4). Among the ten isolates, maximum IAA production recorded from four isolates (SP-01 to 04). *Bacillus*, *Paenibacillus* and *Pseudomonas* strains were isolated from saline and non-saline soil showed a wide range of PGP traits and exhibited maximum phosphate solubilization (Lamizadeh *et al.*, 2016).

Table.1 Biochemical characteristics of bacterial isolates

Sl.No.	Biochemical characteristics	SP-01	SP-02	SP-03	SP-04
01	Gram reaction	+	+	+	+
02	Shape	Bacilli	Bacilli	Bacilli	Bacilli
03	Motility	Motile	Motile	Motile	Motile
04	Growth at 5% Nacl	-	-	-	-
05	Catalase	+	+	+	-
06	KOH Solubility	-	-	-	-
07	Oxidase	+	+	+	+
08	Indole	-	-	-	-
09	Methyl red	+	+	+	-
10	Voges Prauskeur’s test	-	-	-	-
11	Citrate	+	-	+	-
12	Nitrate	-	+	+	+
13	Gelatin	+	+	+	+
14	Starch hydrolysis	-	-	-	+
15	Glucose fermentation	+	+	+	+
16	Mannitol fermentation	+	+	+	+
17	Sucrose fermentation	-	-	-	-

Fig.1 and 2 Rhizosphere isolates nitrogen fixation on NFb medium and zone of colouration in mm

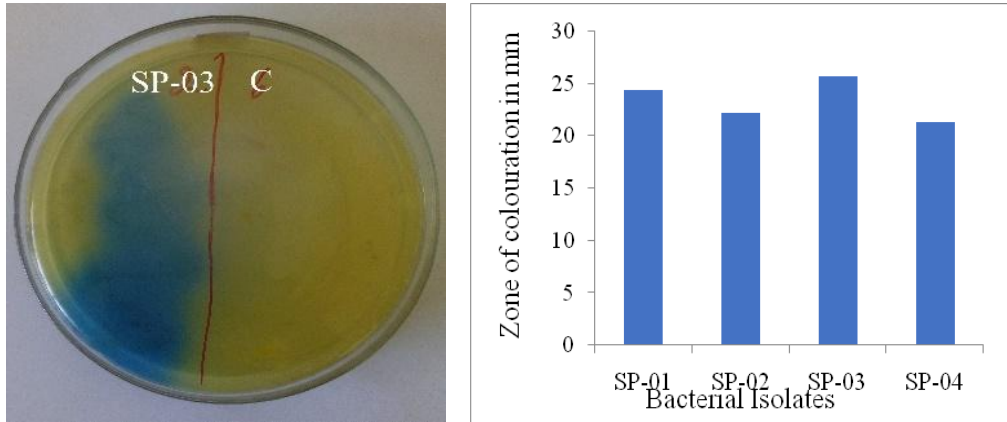


Fig.3 Phosphate solubilization on Pikovskaya's agar medium (Zone of clearance in mm)

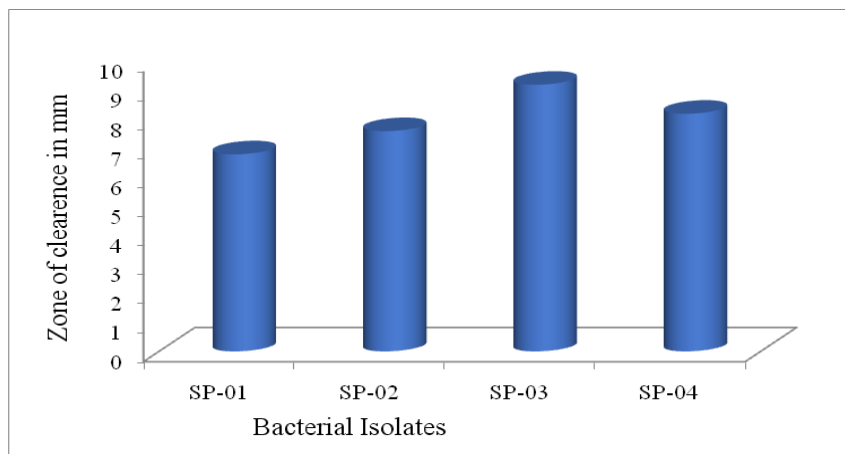


Fig.4 IAA production by Salkowsky's method (pink colour development)

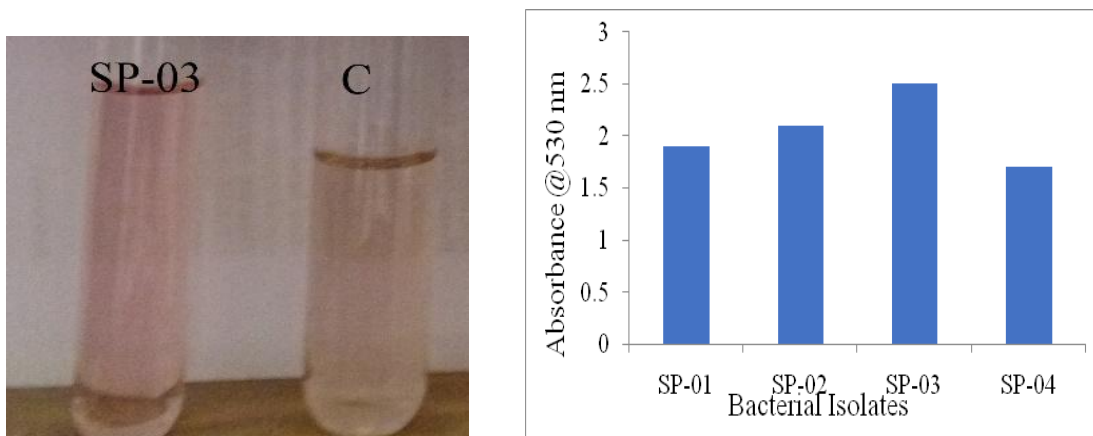
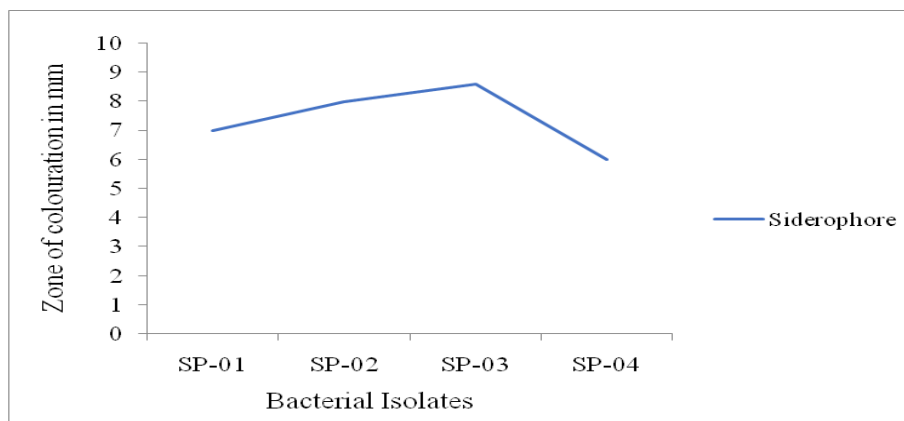


Fig.5 Siderophore production on CAS-HDTMA MM9 medium



Bacterial isolates produced orange halos around the colonies on blue were considered as positive for siderophore production. In this study, there are 7 isolates showed siderophore production positive however, maximum halos observed in isolate SP-03 followed by SP-02 (Figure 5). Siderophore producing *Bacillus* isolates also exhibited different PGPR activities and enhances the plant growth in vegetable plants (Pahari and Mishra, 2017).

In conclusion, plant growth promoting bacteria play a variety of functions in the growth and development of plant as well as crop yield increases in agriculture. In this study, PGPR isolate SP-03 showed maximum PGP traits in *in-vitro* condition perhaps used as a plant growth promoter in agriculture crops. Along with SP-03, other 3 isolates (SP-01, 02 and 04) were also exhibited slight less activity of PGP traits. All these four bacterial isolates used as a bio-fertilizer in various crops after proper validation. Only few studies were reported on PGPR traits of *Brevibacillus* spp., among these studies present report also flood a lime light on PGP Traits of the same spp.

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