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Functional Characterization of Potato Rhizosphere Bacteria *Bacillus cereus* SP4 for Biofertilizer Formulation

Subhadeep Dutta, Sangram Sinha

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ABSTRACT

The present study aimed to isolate potential Plant Growth Promoting Rhizobacteria (PGPR) from potato plant (*Solanum tuberosum* L.) rhizosphere. The isolated bacterial strain SP4 showed IAA production, HCN production, Exopolysaccharide (EPS) production and biocontrol activities against potato fungal pathogens, *Phytophthora* and *Fusarium.* The strain also exhibited good root colonising efficiency in pot trial experiment. The bacterial strain increased plant growth parameters such as shoot length, root length, root number, shoot and root fresh weight, shoot and root biomass, chlorophyll content significantly as compared to untreated control through successful root colonisation. Biochemical characterization and phylogenetic analysis using 16S rRNA gene identified the strain SP4 to be a strain of *Bacillus cereus.* The bacterial strain *Bacillus cereus* SP4, therefore, may be exploited for effective biofertilizer formulation for field application, a goal for reduced chemical fertilizer utilization leading to sustainable agricultural practice.

Keywords PGPR, IAA, Exopolysaccharide, *Bacillus cereus,* Biofertilizer.

Subhadeep Dutta
Department of Botany, Vivekananda Mahavidyalaya, Haripal, Hooghly 712405, WB, India
Email:sinhasangramvm@gmail.com
Corresponding author: Dr Sangram Sinha, Assistant Professor, Department of Botany, Vivekananda Mahavidyalaya, Haripal, Hooghly 712405, WB, India

INTRODUCTION

Potato is one of the essential horticultural commodities in India. International year of potato (2008) declared potato as 4th most important crop among the other crops owing to its food values and consumption in the world. Potato cultivation is a challenging practice because it needs a huge quantity of fertilizers. Therefore, application of huge fertilizer is not only costly but detrimental to the soil health. Soil microbiome is a plethora of natural wealth that actively involved in maintaining ecological balance and nutrient recycling in soil. Rhizosphere bacteria that colonise in the plant roots and enhance plant growth are termed as plant growth promoting rhizobacteria (PGPR) (Vacheron *et al.* 2013). PGPR can influence the plant growth and plant health by various direct and indirect mechanisms. PGPRs can fix atmospheric nitrogen, solubilize phosphate and can produce phytohormones like auxin, gibberellins and can synthesise ACC deaminase. PGPRs also produce HCN, siderophore and antibiotics to fight against potent plant pathogen (Basu *et al.* 2021). PGPR have brought about new bio-revolution for crop production and many micro-

organisms belonging to the genera like *Azospirillum, Bacillus, Burkholderia, Streptomyces, Rhizobium, Erwinia, Azotobacter, Agrobacterium* are now being used commercially (Backer *et al*. 2018). PGPR has been found to be very effective as biocontrol agent as reviewed by many scientists (Grover *et al.* 2021). Many strains of *Bacillus* and *Pseudomonas* have been identified as good biocontrol agent (Khan *et al.* 2018).

Rhizophere engineering using PGPR for sustainable potato production has been a focus research area for many scientists in recent times. Although experimental data regarding application of PGPR for potato cultivation is accumulating in the literature very fast but their commercial application is still very limited. The present study therefore, intended to screen potential PGPR from the rhizosphere of previously unexplored highly productive potato fields of North Hooghly District of West Bengal, India and its possible application as biofertilizer.

MATERIALS AND METHODS

Isolation of PGPR from potato rhizosphere : Soil samples were collected from the rhizosphere soil of potato plants in different potato fields of Hooghly District in West Bengal, India. Ten grams of air-dried samples were used for dilution plating and plates were kept for 3 days at 30ºC. Typical bacterial colonies were observed into the plate. Well isolated bacterial colonies were selected on the basis of difference in colony morphology and streaked into nutrient agar slants.

HCN production : The bacterial isolates were streaked on King's B medium amended with glycine at 4.4 g/lit. A sterile filter paper was placed in the lids of each Petri dishes saturated with picric acid solution (2.5 g of picric acid; 12.5 g of Na_2CO_3, 1000 ml of distilled water). Then the Petri plates were sealed with Parafilm and incubated at 28°C for 48 h. A change of colour of the filter paper from yellow to light brown, brown or reddish-brown confirmed HCN production.

ACC deaminase activity : Screening for ACC deaminase activity of the bacterial isolates were carried out by streaking to ACC amended (3 mM ACC as nitrogen source) modified DF (Dworkin and Foster) salts minimal medium. Appearance of growth confirmed the efficiency of the isolates to utilize ACC as nitrogen source in DF minimal agar medium.

Screening of indole acetic acid (IAA) production and quantification under different temperature and pH : Identification of IAA was determined by slightly modified method by Brick *et al*. (1991). For quantification and optimization of IAA production, bacterial cultures were grown in nutrient broth amended with 0.5 mg/ml of tryptophan in shaking incubator for 72 h at three different temperature (30ºC, 37ºC and 40ºC) and in three different pH (5, 6, 7, 8 and 9). After incubation, cultures were centrifuged at 10,000 rpm for 10 min. After that 2 ml of supernatant was taken from each experimental set and mixed with 2 to 3 drops of orthophosphoric acid and 4 ml of Salkowski reagent (50 ml 35% perchloric acid, 1 ml 0.5 M FeCl3 solution). Development of pink color indicated IAA production. Quantitative estimation of IAA production was done by measuring the OD at 530 nm with the help of spectrophotometer using IAA standard curve (10-100 µg/ml).

Screening of EPS production and quantification : The bacterial isolates were streaked to yeast extract mannitol agar and grown for 30ºC for 48 h for screening EPS production following the method of Sayyed *et al*. (2015) with little modification. Appearance of mucoid colony confirmed positive test. For quantitative estimation, one loop full of overnight grown bacterial culture was inoculated to 50 ml yeast extract-mannitol broth (YEMB) at three different temperature treatment for 3 days with constant shaking at 120 rpm. Viscosity of the culture were monitored daily. After 3 days the bacterial cultures were centrifuged (10000 rpm, 20 min) and the supernatants were collected for EPS estimation. Equal volume of 30% (v/v) isopropanol was slowly added to the supernatant with constant stirring and EPS was separated by spooling. Spooled samples were oven dried at 50ºC until a constant weight and was obtained for estimation of EPS.

Antifungal activity (Dual culture methods) : Antifungal activities were tested against two fungal pathogens, *Fusarium* sp. and *Phytophthora* sp. that had been collected from diseased potato tuber from

the fields of Haripal block, WB, India in this study. Antifungal activities were tested following dual culture method (Berg *et al.* 2005). The percentage of inhibition was measured according to standard procedure.

Potato root colonization assay : Potato root colonisation by eight selected bacterial isolates were carried out using rifampicin resistant (250 µg/ml) spontaneous mutants on nutrient agar (NA) media. Roots of the potato stem cuttings were immersed in overnight grown bacterial cell suspension (10^8 cfu/ml) for 3 h. For each bacterium, ten plants were bacterized and grown in sterilized plastic pots. Rhizosphere soils were collected and analyzed for recovery of rifampicin resistant bacteria by dilution plating in rifampicin (250 µg/ml) added NA plates after 5, 15 and 30 days

Pot assay for potato growth promotion and tuber yield by the strain SP4 and VM2

Two strains, SP4 and VM2 that showed maximum colonization efficiency were finally chosen for pot assay. Potato tuber seeds having about 4-5 eyes were purchased from local market at Sheoraphully, WB, India and were surface sterilized by washing in 20% ethanol for 30 seconds followed by shaking with 1% sodium hypochlorite, 0.1% Tween-20 solution for 10 minutes. The tubers were then washed thoroughly with autoclaved distilled water. Single eye potato seed pieces were dipped in young bacterial suspension of SP4 and VM2 (2×10^9 cfu/mL) for 3 h. Two seeds from each treatment were then planted in each sterilized plastic pots (15 cm height and 18 cm diameter) filled with sterilized soil. For control set single eye potato seed pieces was dipped in sterilized water. Plants were harvested after five weeks. Growth parameters such as length, fresh weight, dry weight, for root and shoot were recorded. For dry weight measurement, plant parts were oven dried at 60°C till constant weight. The chlorophyll content of the mature leaves was determined (Sinha and Mukherjee 2008). The experiments were carried out in triplicate.

Morphological and biochemical characterization of bacterial isolate SP4 : The morphological characteristics of the bacterial colonies were observed on nutrient agar plates. All the isolates were streaked onto the nutrient agar plates. The size, shape, elevation, surface, margin, color, pigmentation of the colonies were recorded. Gram stain characterizations were performed by standard gram stain method. Taxonomic identification of the bacterial strains isolated from soil will be carried out by Gram staining and standard biochemical tests.

Molecular characterization of the bacterial isolate SP4 by 16S rDNA gene sequencing and phylogenetic tree analysis : Genomic DNA of the selected strain was extracted as per standard procedure. Amplification of 16S rDNA was performed from the genomic DNA using universal primers (Weisburg *et al.* 1991), fD1 (5′ -GAGTTTGATCCTGGCTCA–3′) AND Rp2 (5′- ACGGCTACCTTGTTACGACTT –3′). The sequencing of the PCR product was done by SciGenom, India. To perform phylogenetic analysis, reference sequences required for comparison were downloaded from NCBI database. All the sequences of 16S rRNA were aligned using the multiple sequence alignment program CLUSTALW (Larkin *et al.* 2007) and Phylogenetic tree was constructed using MEGAX software (Kumar *et al.* 2018). The evolutionary history was inferred by using the Maximum Likelihood method.

Statistical analysis : Data were analyzed statistically for different important parameters using one-way ANNOVA at $p<0.05$ and Fisher's least significant difference (LSD) at $p=0.05$ were calculated to determine statistically significant difference between the mean values.

RESULTS AND DISCUSSION

Bacterial strains that were isolated from potato rhizosphere were screened on the basis of colony morphology. Light microscopic observation and preliminary biochemical characterization of hundred bacterial isolates (date not shown) revealed the predominance of different gram-positive bacterial strains, tentatively *Bacillus* strains in potato rhizosphere with only very few gram-negative speculative, *Pseudomonas* strains, On the basis of growth and PGPR characteristics such as Phosphate solubilization, HCN production, IAA production, , ACC utilization and Exopolysaccharide synthesis (EPS), eight best isolates viz., MS1, MS2,

Table 1. PGPR characterization of eight bacterial isolates. Bacterial strains showing positive result for each test indicated by + sign and negative test by – sign respectively..

Bacterial strain	Phosphate solubilization	HCN production	IAA production	ACC deaminase	EPS production
MS1	-	-	+	-	+
MS3	-	+	+	-	+
MS4	-	-	+	-	+
MS5	-	-	+	-	+
SP4	-	+	+	+	+
SP12	-	-	+	-	+
SP13	-	+	+	-	+
VM2	-	+	+	+	+

MS3, MS4, MS5, SP4, SP12, SP13, VM2 were selected for further study. Two isolates, SP4 and VM2 were found to exhibit maximum positive response that included positive response for HCN production, IAA production, ACC utilization and exopolysaccharide synthesis (Table 1).

Plant microbe interaction in the rhizosphere is essential for sustainable plant ecology and any alteration may severely affect plant productivity. PGPR has been reported to modulate plant growth response in number of different ways (Cheng *et al.* 2019). However, the exact mechanism is very complex and remained undisclosed. Recent research on application of PGPR have drawn considerable interest and accumulation of data of pot-based experiments and field trials certainly encouraged the scientists to conceptualise biofertilizer formulation using PGPR and its possible commercialisation (Backer *et al.* 2018). IAA is the product of tryptophan metabolic pathway and is the most potent physiologically active auxin responsible for hormonal homeostasis in plants. IAA synthesis by many rhizosphere bacteria have been reported to improve plant growth and the amount of IAA synthesised may vary depending on the cultural condition and bacterial species (Mohite 2013, Chandra *et al.* 2018). The study, therefore, intended to examine the efficacy of bacterial isolate to synthesize auxin under tryptophan amended cultural condition. Out of the eight isolates, two bacterial isolates SP4 and VM2 were found to be most efficient IAA producing strains. The optimal production of IAA by SP4 and VM2 were15.3 ug/ml and 10.44 ug/ml respectively (Fig.1) corroborating with the previous workers where they had reported varying IAA production by *Bacillus* sp. (Chagas *et al. 2*015). The optimum temperature for IAA production, however, found to differ among different bacterial isolates and reported to be maximum at 37ºC by Chandra *et al.* (2018) and 35ºC by Kumari *et al.* (2018), whereas others also observed maximum IAA production at 30ºC (Mohite 2013). All the strains in this study exhibited maximum IAA production of at 30ºC in concert with previous reports. Among the when IAA production was studied using different pH in the media, the maximum IAA

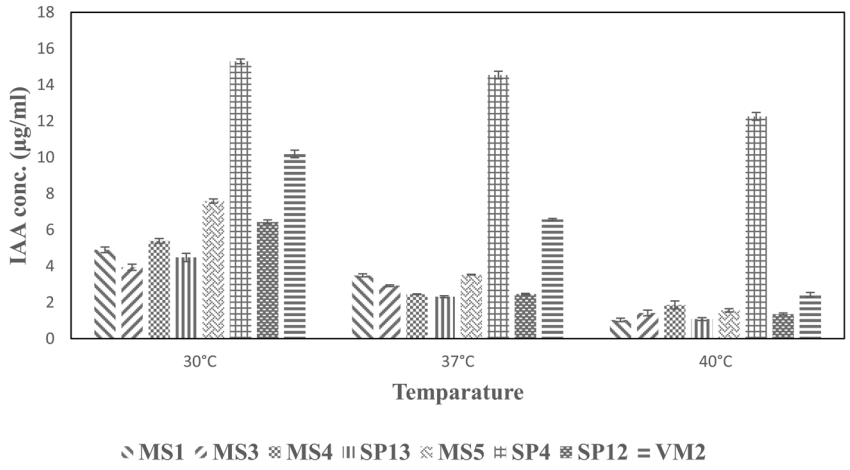

Fig. 1. Effect of Tem on IAA production by bacterial isolates.

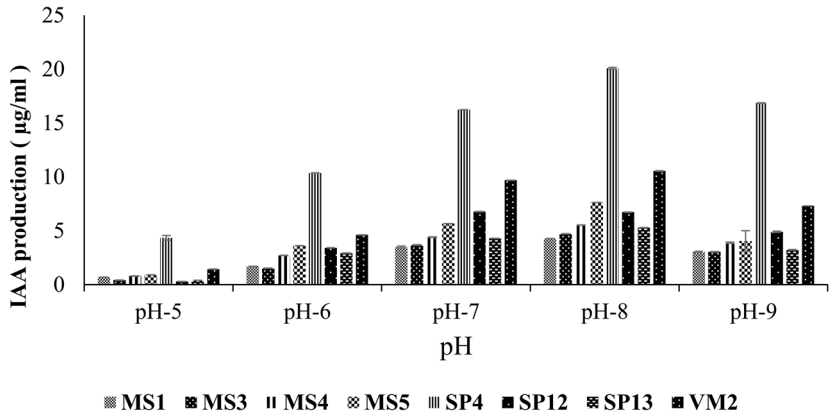

Fig. 2. Effect of pH on IAA production by bacterial isolates.

production was noticed in the alkaline media by all the eight bacterial strains having pH 8.00 followed by pH 7 and acidic pH inhibited IAA production. The results are concomitant with the earlier researchers who also reported maximum IAA production in pH 8.00 (Yousef 2018). The bacterial isolates SP4 and VM2 were found to produce 17.1 ug/ml and 9.59 ug/ml IAA respectively at pH 8.00 (Fig. 2).

Exopolysaccharides are complex saccharide polymers synthesised by many bacteria (Ghosh and Maity 2016) belonging to the genera *Pseudomonas, Bacillus, Ralstonia, Rhizobium, Azotobacter*. This complex polysaccharide encompasses variety of mechanism that are beneficial for the growth and development of plants (Costa *et al.* 2018). Eight selected bacterial isolates had been tested for optimum EPS production under different temperature treatment. The bacterial isolate SP4, exhibited highest EPS production of 2.59 mg/ml dry weight at 37ºC followed by VM2 producing 1.44 mg/ml at 37ºC (Fig. 3). The result was consistent with the earlier report by Mu'minah *et al.* (2015) where EPS production by bacterial isolates from potato rhizosphere varied between 0.10 mg/ml dry weight to 2.24 mg/ml dry weight. The all-other isolates produced maximum EPS at 37ºC, however, the amount of EPS production was very less. The optimum temperature for EPS production by different *Bacillus* sp. has been reported to be 37ºC (Solmaz *et al.* 2018) which correlated our

Table 2. Root colonization study using Rifampicin resistant mutant strains of the bacterial isolates. Data are the mean of three replicates with SE.

Bacterial strains	Root treatment with bacteria 10^8 CFU/ml (log cfu)	Recovery of rifampicin resistant Bactria (Log cfu/g of rhizosphere soil)		
		5 days	10 days	15 days
MS1	8	2.534 ± 0.00666	Not Detected	Not Detected
MS3	8	2.464 ± 0.008181	Not Detected	Not Detected
MS4	8	2.367 ± 0.002152	Not Detected	Not Detected
MS5	8	2.483 ± 0.011116	Not Detected	Not Detected
SP4	8	6.935 ± 0.006991	6.791 ± 0.007485	6.810 ± 0.00216
SP12	8	2.521 ± 0.002717	Not Detected	Not Detected
SP13	8	2.464 ± 0.003469	Not Detected	Not Detected
VM2	8	2.540 ± 0.003639	2.159 ± 0.006092	Not Detected

Table 3. Plant growth promotion in potato *(Solanum tuberosum)* by SP4 and VM2. Data for each treatment regime are the mean of 5 replicates with ± SE. Values with superscript of different letters in each column represent significant difference from one another at p=≤0.05 and same letters are insignificant at p=≤0.05.

Treatments	Shoot length (cm)	Root length (cm)	Root number	Shoot fresh Wt. (g/plant)	Shoot biomass (g/plant)	Root fresh weight (g/plant)	Root biomass (g/plant)	Chlorophyll content (mg/g fresh leaf)
Control (A)								
	17.78 ^{a} ±0.91	12.34^{a} ±1.05	6.8 ^{a} ±0.58	41.68 ^{a} ±1.64	6.12 ^{a} ±0.75	9.04 ^{a} ±1.16	1.72 ^{a} ±0.15	2.28 ^{a} ±0.13
Control +SP4								
	21.54 ^{b} ±1.01	20.2b^{c} ±0.34	11.8^{bc} ±0.86	54.48 ^{bc} ±0.60	11.66^{bc} ±0.25	14.2^{bc} ±0.63	4.1^{bc} ±0.13	3.1^{bc} ±0.11
Control+VM2								
	16.04 ^{a} ±0.46	12.54^{ac} ±3.42	6.8^{ad} ±1.13	38.84 ^{ad} ±3.24	6.64^{ad} ±1.60	7.36 ^{ad} ±1.50	1.34 ^{ac} ±0.12	1.82 ^{ad} ±0.36
LSD value at 5%	2.56	6.40	2.99	6.55	2.00	2.70	1.01	1.09

observation of maximum EPS production at 37ºC.

Two bacterial isolates SP4 and VM2 had shown antifungal activity against *Fusarium* and *Phytopthora* in dual culture (Fig. 4). The maximum antifungal activity was 82% exhibited by both the isolate against *Fusarium*. However, the inhibition activities by SP4 and VM2 were much less and around 66% and 62% respectively against *Phytophthora.* It has been reported that many PGPR including *Bacillus* sp. can act as antagonist to fungal pathogens and they possess variety of mechanism to control the growth of fungal pathogens (Calvo *et al.* 2010, Ali *et al*. 2019). EPS production by *Pseudomonas aeruginosa* PF23 have been reported to control plant pathogen in sunflower by Tewari and Arora (2014). HCN production by *Pseudomonas* strain have also been reported to control the growth of *Phytophthora infestans* by Anand *et al*. (2020). Therefore, two bacterial isolates SP4 and VM2 might have adapted any of the mechanisms for controlling the growth of the phytopathogens which is needed to be investigated further. The present result commensurate with the report of earlier researchers that *Bacillus* sp. could control the growth of *Fusarium*

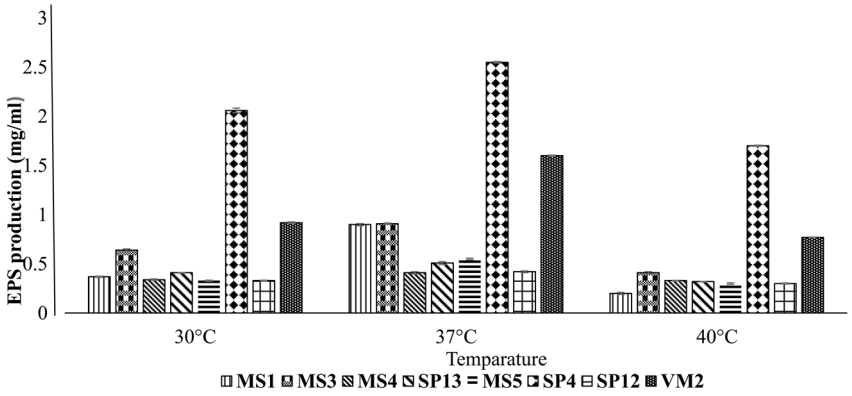

Fig. 3. Effect of Tem on EPS production by bacterial isolates.

(Khan *et al.* 2018). However, further research is required to uncover the exact mechanism of biocontrol by SP4 and VM2.

Biofilm formation and exopolysaccharide (EPS) synthesis by PGPR is pivotal and plays a decisive role in successful root colonization (Knights *et al.* 2021). EPS deficient mutant analysis of *Bacillus pumilus* HR10 revealed that the mutation hindered colonization of the mutant strain in *Pinus thunbergia* (Zhu *et al.* 2020). In view of the above observation, the ability of EPS producing bacterial isolates to colonize in the root rhizosphere of potato had been evaluated. It had been observed that highest RPS producing bacterial strain SP4 had shown maximum efficiency for root colonization followed by the bacterial strain VM2 (Table 2).

Pot based experiment revealed that there is a clear trend of growth promoting activity in potato by bacterial isolate SP4 when applied as bioinoculant. However, bacterial isolate VM2 although sharing many traits like SP4 failed to enhance plant growth when compared to the control plant for most of the parameters. Bacterial isolate, SP4 enhanced shoot length, root length, number of roots, shoot and root fresh weight, shoot and root biomass and chlorophyll content significantly over the untreated control when analyzed by one-way ANNOVA and the p value calculated were found to be well below 0.05 (Table 3). The bacterial isolate improved root fresh weight and root biomass by 57.07% and 138.37% respectively. This result may be explained by the fact that IAA produced by the bacterial strain might have stimulated root initiation as IAA have been reported to induce root initiation in plants (Aloni *et al.* 2006). IAA induced root initiation by SP4 might have facilitated water and nutrient uptake in potato plants thereby

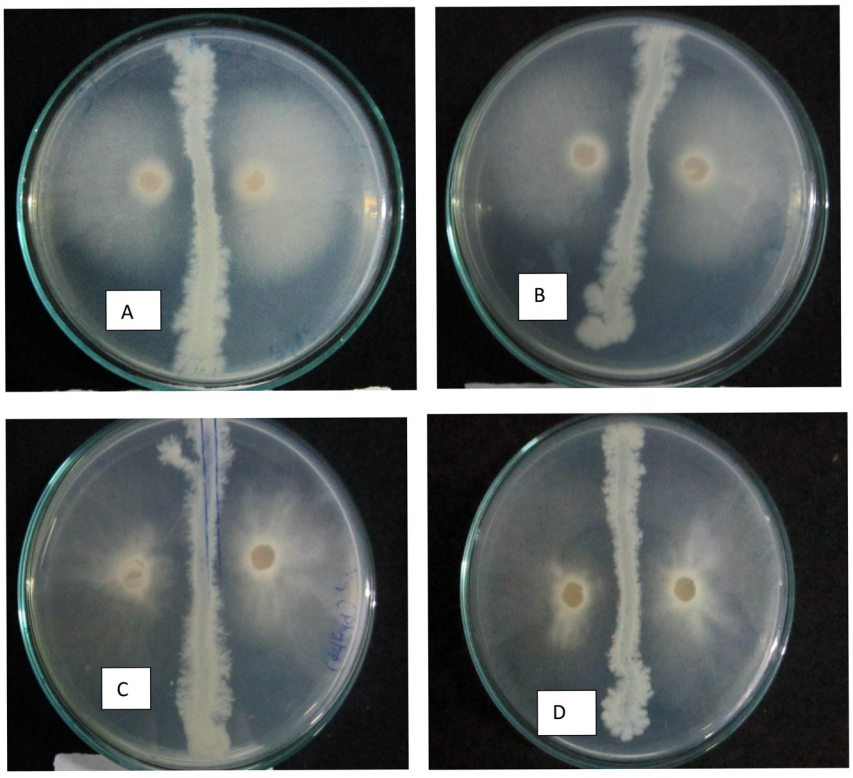

Fig. 4. Antifungal activities of SP4 and VM2 against *Fusarium* sp. represented by A and B respectively and against *Phytophthora* sp. represented by C and D respectively in dual culture.

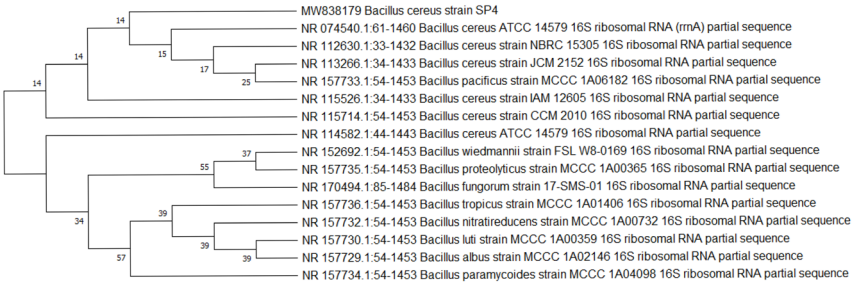

Fig. 5. Phylogenetic tree showing evolutionary relationship between the isolated strain and matching strains from NCBI database. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

improving its vigour and health. Bacterial IAA might have efficiently absorbed by the root and translocated through the xylem to the aerial part and increased shoot length by stretching the cells (Arkhipova *et al.* 2020). The bacterial isolate VM2 although apparently showed IAA production ability in Salkowsky reagent, however, the confirmative test of IAA was not done. Therefore, it is probably a false positive result for IAA production and this might have been reflected in the pot experiment where VM2 failed to promote plant growth. Exopolysaccharide produced by PGPR benefits plants in many diverse ways and specially under stress (Mokrani *et al.* 2020). The bacterial isolate SP4 is a good EPS producing strain and a successful coloniser in the potato rhizosphere as observed in this study. EPS production capability of SP4 might have offered advantages to the host plants by forming soil aggregates important for trapping water and nutrients and also possibly through increasing soil enzyme activities (Alami *et al.* 2000).

Table 4. Biochemical characterization of bacterial isolate SP4. Bacterial strains showing positive result for each test indicated by + sign and negative test by – sign respectively.

Morphological and biochemical characterization	Bacterial strain SP4
Margin	Irregular
Consistency	Slimy
Gram staining	+
Indole	-
Methyl Red	-
Voges Proskeur	+
Citrate	+
Catalase	+
Oxidase	-
Starch Hydrolysis	+
Casein Hydrolysis	+
Urea Hydrolysis	+
Gelatin Hydrolysis	+
H_2S Production	-
Nitrate Reduction	-
Dextrose	+
Mannitol	-
Xulose	-
Sucrose	+
Comment	*Bacillus* sp.

The bacterial isolate SP4 was characterized morphologically and biochemically and appeared to be a species of *Bacillus* (Table 4). Phylogenetic analysis of 16S rRNA gene (Gene Bank accession Number MW 838179) revealed that the strain showed 100% homology with *Bacillus cereus* (Fig. 5).

The present study, therefore, concluded that IAA and EPS producing bacterial strain, *Bacillus cereus* SP4 is a potential plant growth promoting bacteria and it may be used as biofertilizer for improving potato plant vigour and as biocontrol agent to control pathogenic attack. However, further studies may require to analyse its efficacy under actual field condition with proper formulation using suitable carrier material.

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