ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Effect of polyhydroxyalkanoates accumulated plant growth promoting *Bacillus* sp. on germination and growth of Mung Bean & Groundnut.

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ABSTRACT

Polyhydroxyalkanoates (PHA) accumulation is important feature that enhances shelf life of plant growth promoting microbes which play a crucial role for production of bio inoculants with suitable and sustainable bacterial cells as a carrier for agricultural use. Recent research has elucidated key properties of few *Bacillus* species identified biochemically that contribute production of siderophore, ammonia, synthesis of IAA and solubilization of inorganic phosphate and their ability to promote growth of plant. Here qualitative and quantitative data of PGPR characteristics pertaining to the results of seed germination and pot culture. The field trial is clear an observation regarding the healthy growth, preservation of plant from pest as well as property of the soil.

Keywords: Polyhydroxyalkanoates (PHA), IAA, Siderophore, PGPR

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ISSN: 0975-8585

INTRODUCTION

Unsatisfactory food safety issues, increasing environmental food concerns and status of plant available nutrients are gaining more attention towards biofertilizer and biopesticides agriculture sector world-wide. Plant Growth Promoting Rhizobia (PGPR) having Polyhydroxyalkanoates (PHA) accumulating capability enhances the progression of roots and plants by secreting extracellular metabolites. Their hidden or untapped reservoir has antagonistic effect against pest management. It has been clearly elucidated that rhizosphere has hidden or untapped reservoirs for PHA accumulators in addition to PGPR and antagonistic effect. Storage and utilization of polyhydroxyalkanoates are important for the shelf life enhancement of the bacteria in production of bioinoculants, products containing bacterial cells in a suitable carrier for agricultural use. Production of PHA holds advantageous characteristics towards enhanced root colonization, plant growth promotion, survivability, chemotaxis, motility, and cell multiplication. Based on cultivation-dependent methods, earlier it was reported that rhizosphere has low PHA production in comparison to non-rhizosphere soil [1] Cultivation-independent and molecular techniques allowed us to conclude that wheat, oilseed rape, and sugar beet rhizosphere have more PHA production [1]. Plant growth promoting rhizobacteria (PGPR) are functionally diverse group of bacteria having immense potential as biofertilizers and biopesticides. Depending upon their function, they may serve as partial replacements for chemical fertilizer or pesticides as an eco-friendly and cost-effective alternative as compared to their synthetic counterparts. Therefore, isolation, characterization and practical evaluation of PGPRs having multifaceted beneficial characteristics, are essentially required. This study describes the detailed of *Bacillus* sp. S4 and S8 PHA accumulating bacterial isolates having plant growth promoting traits.

MATERIALS AND METHODS

Sample collection, Isolation and preservation of bacterial isolates

Identified Bacterial isolates as *Bacillus* sp. S1, *Bacillus* sp. S4, *Bacillus* sp. S6 and *Bacillus* sp. S8 were revived using Nutrient Broth medium (Hi-media laboratories private limited, Mumbai) which was previously isolated from Sugarcane rhizospheric region. **A**fter two years of preservation these isolates were again screened for presence of PHAs granules by Sudan Black B staining. Prior to staining bacterial isolates were inoculated to GM medium for induction of PHA granules [2] (Mohapatra *et al*., 2014).

Screening and Qualitative analysis of PHAs accumulating isolates for different plant growth promoting activities

The bacterial isolates were screened for different plant growth promoting activities. Primarily the isolates screened qualitatively for phosphate solubilization, auxin (IAA), ammonia and siderophore production. Isolates were further screened for their HCN producing abilities to ensure as these organisms are having PGPR activity [3].

Quantification of IAA production and phosphate solubilisation by the PGPR isolates

Quantitative estimation of phosphate

Quantitative estimation of inorganic phosphate solubilization was done as per methodology described by Mehta and Nautiyal, 2001 [4]. Bacterial isolates were grown in National Botanical Research Institute's Phosphate (NBRIP) broth containing 0.5% tricalcium phosphate (TCP). The absorbance of the resultant color was read after 10 min at 430 nm in UV/Visible Spectrophotometer. The total soluble phosphorus was calculated from the regression equation of standard curve and expressed as µg ml^{-1} over control. The pH of culture supernatant was also measured using a pH Meter.

Quantitative estimation of IAA

Quantitative estimation of IAA was done as per methodology explained by Gordon and Weber, 1951[5]. Estimation of indole-3-acetic acid (IAA) in the supernatant was done by spectrophotometer. 1ml of supernatant was mixed with 4 ml Salkowski reagent and absorbance of the resultant pink color was read after

30min at 535 nm in UV/Visible Spectrophotometer. The IAA production was calculated from the regression equation of standard curve and the result was expressed as µg ml^{-1}over control.

Seed germination test

After this in-vitro analysis, two different experiments were conducted such as roll towel and pot culture method to check the germination of seeds and growth of plants.

Roll towel method

The potential PHAs isolates S4 and S8 showing plant growth promoting activities were tried with Mung bean (variety: Pragya) and Groundnut (Smruti). The seeds were collected from Plant breeding department, OUAT. The seeds were surface sterilized with 0.1% $HgCl_2$ for 2 min and rinsed with sterile distilled water. Bacterial isolates were grown in nutrient broth on shaking incubator (180 rpm) at 28 ± 2°C for 24 h. Cell densities in the suspension were adjusted to a final density of approximately 10^8 CFU ml^{-1}. The surface sterilized seeds of Mung bean were inoculated in broth culture for 30 minutes. Germination tests were carried out by the paper towel method and PGPR-treated seeds and control were seeded onto paper towels.

Pot Culture Method

Pot culture method conducted where each pot contains 3 kg of soil which were collected from OUAT experimental field and watered in (1:2 w/v) soil: water ratio. For this experiment Mung bean and Groundnut Seeds were soaked in inoculum for 30 minutes and were sown at 2 cm depth in pot. A control (untreated seed) was also maintained in the experiment. Germination percentage was measured with following formula:

Germination percentage = Number of germinated seeds / Number of seeds in sample × 100.

Physical and Chemical properties of soil

To validate the potency of the bacterial PGPR activity field experiment was conducted where various analysis were done to evaluate strength of the soil before and after field experiment (after treatment). Analysis of physico-chemical properties such as organic carbon (g/kg), pH, Electrical conductivity (ds/m), available N (kg/ha), P_2O_5(kg/ha) and K_2O (kg/ha) of Mung Bean and Groundnut field soil was carried out in the Soil chemistry department, O.U.A.T, Bhubaneswar.

Field trial

The potential PHA accumulated *Bacillus* species S4 and S8 showing good PGPR activities was simultaneously tried with Mung bean and Groundnut for determination of effect on plant growth and crop productivity under field and natural environmental conditions. These experiments were carried out in triplicate and data was analyzed statistically by one-way ANOVA using SPSS 16.0 software in addition the significance of differences between mean values was evaluated by DMRT (Duncan's Multiple Range Test).

RESULTS AND DISCUSSION

Plant growth promoting activities of the PHA producing bacterial isolates

In search of efficient PHAs accumulator with plant growth promoting activites, the four bacterial isolates were screened for phosphate solubilization on Pikovaskya agar, among four isolate S4 and S8 showed sharp halo zones (Table 1, Figure 1). In quantitative estimation of phosphorus, tri calcium phosphate was taken as standard and S4 and S8 produced 6.3 and 9.2 µg/ml of phosphorus respectively (Table 2). Similar results were also observed by Rodriguez *et al*., 1999 [6] who reported that sharp halo zone is the indicative of phosphate solubilization. These two isolates were also able to produce Siderophore whereas iron is an important growth element for all living organisms [7]. Growth promotion attributed to other mechanisms such as production of phytohormone Auxin that promotes plant growth [8]. In this context the PHA producing *Bacillus* sp. S8 also produced plant growth promoting hormone i.e. IAA. (Table 1, Figure 1). In quantitative estimation of IAA among four isolate, S8 was found to produce highest 130.2 µg/ml of and S4 produce 82.6

ISSN: 0975-8585

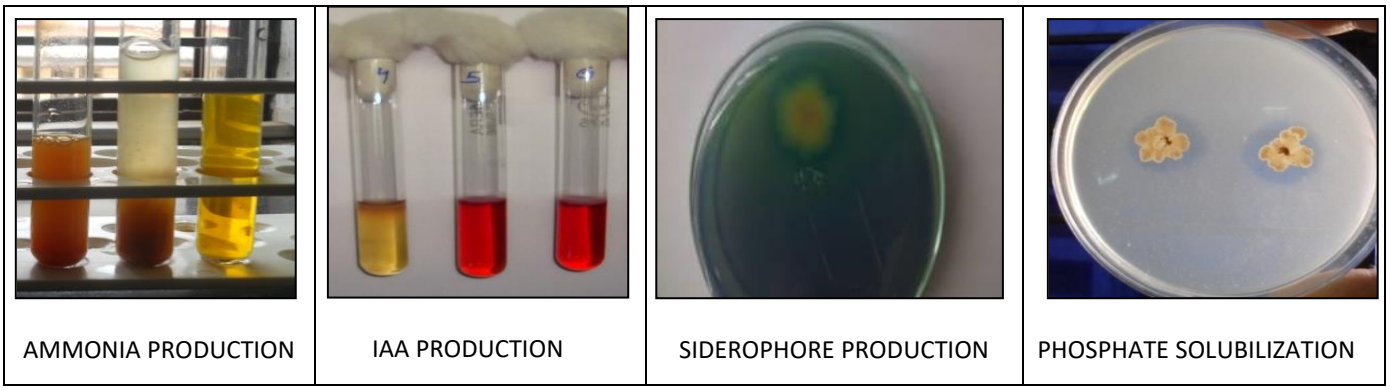
µg/ml of IAA (Table 2). The differences in auxin-production may be attributed to the inherent properties of the individual bacteria towards PGPR activity [9]. This result depicted *Bacillus* sp. S4 and S8 exhibited strong production of ammonia, which is taken up by plants as a source of nitrogen for their growth (Table 1) similar result was also driven by Ahmad et al., 2008 [10].

Table 1: PGPR activities and Antibiosis shown by the PHAs producing bacterial isolates

Isolates	Ammonia	IAA	Siderophore Zone size	Phosphate Zone size	*Rhizoctonia* sp. (ITCC 186)	*Alternaria solani* (ITCC 3640)	*Aspergillus* sp. (ITCC 4065)
***Bacillus* sp. S1**	-ve	+ve	15 mm	20 mm	0 mm	0 mm	0 mm
***Bacillus* sp. S4**	- ve	-ve	10 mm	15 mm	0 mm	15 mm	0 mm
***Bacillus* sp. S6**	+ ve	-ve	12 mm	18 mm	22 mm	0 mm	10 mm
***Bacillus* sp. S8**	+ ve	+ve	20 mm	22 mm	22 mm	24 mm	24 mm

-ve: Negative, +ve: Positive, mm: millimeter

Fig 1: PGPR Test results



AMMONIA PRODUCTION | IAA PRODUCTION | SIDEROPHORE PRODUCTION | PHOSPHATE SOLUBILIZATION

Table 2: Quantitative Analysis of IAA and Phosphate Solubilization

Isolates code	Phosphate Solubilization (µg/ml)	IAA production (µg/ml)
***Bacillus* sp. S1**	5.0 ± 0.032^{a}	8.25 ± 0.197^{b}
***Bacillus* sp. S4**	5.5 ± 0.031^{ab}	3.13 ± 0.175^{a}
***Bacillus* sp. S6**	6.3 ± 0.445^{b}	82.6 ± 1.478^{c}
***Bacillus* sp. S8**	9.2 ± 0.280^{c}	130.2 ± 1.816^{d}

*Values are the mean ± SEM and differ significantly as per DMRT by LSD (P <0.05). Mean values in each column with same superscript (s) do not differ significantly as per DMRT.

Antibiosis

The bacterial isolates were tested for antibiosis against three common plant pathogen *Aspergillus* sp., *Alternaria solani* and *Rhizoctonia solani*. *Bacillus* sp. S8 showed significant halo zone that clearly indicating these pathogens are sensitive toward towards the metabolites of *Bacillus* sp. S8 (Table 1). Several studies reviewed that siderophore production by PGPR was most effective in controlling the plant root pathogens [11].

Seed germination test by roll towel method

After qualitative and quantitative analysis pot experiment reveled S8 is more potential and positively affected the germination of mung bean and groundnut seeds over S4 and control (Figure 2 and 3). This study demonstrated an increase in the plant growth by seed bacterization. It is a well-established fact that improved phosphorous nutrition influences overall plant growth and root development [12].

Pot culture method

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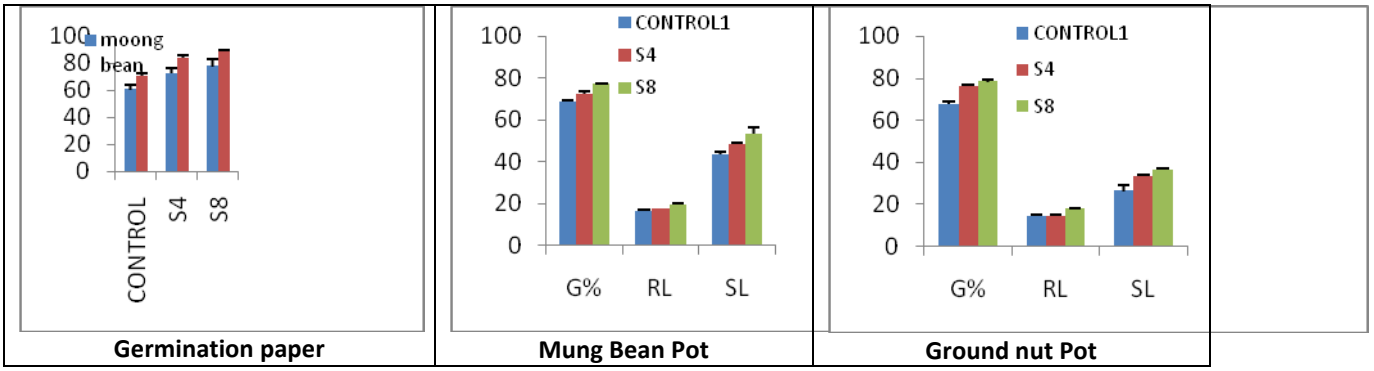
Effectiveness of PHA accumulating PGPR isolates on germination percentage and growth of mung bean and groundnut seeds were evaluated. Seeds those were pretreated with *Bacillus* sp. S8 and S4 it was significantly increased the germination percentage, root and shoot length in both crops as compare to the control. Highest root and shoot elongation of the plant were recorded when seeds were pre-treated with *Bacillus* sp. S8 in pot. All statistical data are shown as mean ± SEM. These results suggest that polyhydroxyalkanoates accumulating *Bacillus* sp. S8 is able to stimulate the production of IAA, Phosphorus Solubilization, Siderophore Production and thereby improving growth of plants. A large number of evidence suggests that PGPR enhance the growth, plant height and biomass [13] (Figure 2 and 4).

Fig 2: Germination and Growth of Mung Bean and Groundnut.

Seed Germination in Roll towel Method		**Germination and Growth in Pot Culture Method**	
Control Mung Bean	**Control Ground Nut**	**Control Mung Bean**	**Control Ground Nut**
			
S4 Mung Bean	**S4 Ground Nut**	**S4 Mung Bean**	**S4 Ground Nut**
			
S8 Mung Bean	**S8 Ground Nut**	**S8 Mung Bean**	**S8 Ground Nut**
			

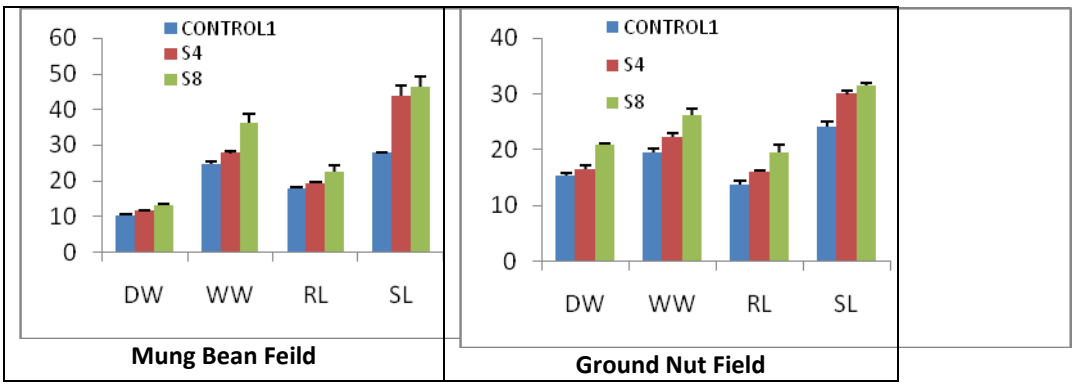
Fig 3: Germination %, Root length and Shoot length of Mung bean and Groundnut

ISSN: 0975-8585



Values are the mean ± SEM and differ significantly as per DMRT by LSD (P <0.05). Mean values in each column with same superscript (s) do not differ significantly as per DMRT.

Fig 4: Dry weight, wet weight, root length and shoot length of Mung bean and Groundnut in field conditions.



Values are the mean ± SEM and differ significantly as per DMRT by LSD (P <0.05). Mean values in each column with same superscript (s) do not differ significantly as per DMRT.

Physical and Chemical properties of soil

After *Bacillus* sp. S4 and S8 inoculum treatment the physical-chemical properties such as Organic Carbon, Available Nitrogen, Phosphorus, Potassium, Soil pH, Electrical Conductivity were also increased significantly from control in Mung bean and Groundnut field from control. All the obtained data comes statistically significant (P <0.05) (Table 3 and 4). From several studies and literature review it has been found that after treatment of PGPR as bio inoculant organic carbon, nitrogen and phosphorus also increases in the soil environment [14] with slight reduction in pH [15]. It has already been reviewed that production of organic acids by soil microorganisms and commensurate pH decrease is the major mechanism of phosphate solubilisation [16].

Table 3: Physical and Chemical properties of soil of Mung bean field

Isolate code	O.C (g/kg)	N (kg/ha)	P_2O_5 (kg/ha)	K_20 (kg/ha)	pH	EC (ds/m)
Control	0.193 ± 0.01 [a]	163.6±0.9 [a]	319.0±0.251 [a]	31.0±0.41 [a]	5.1±0.03 [a]	0.073±0.1[a]
S4	0.194±0.01 [a]	166.2±0.05 [b]	320.0±0.06 [a]	32.0±0.14 [b]	5.4±0.05 [b]	0.074±0.1[a]
S8	0.197±0.01 [b]	169.2±0.12 [c]	321.3±0.08 [a]	34.3±0.08 [c]	5.6±0.03 [c]	0.078±0.1[b]

*Values are the mean ± SEM and differ significantly as per DMRT by LSD (P <0.05). Mean values in each column with same superscript (s) do not differ significantly as per DMRT.

ISSN: 0975-8585

Table 4: Physical and Chemical properties of soil of Mung bean field

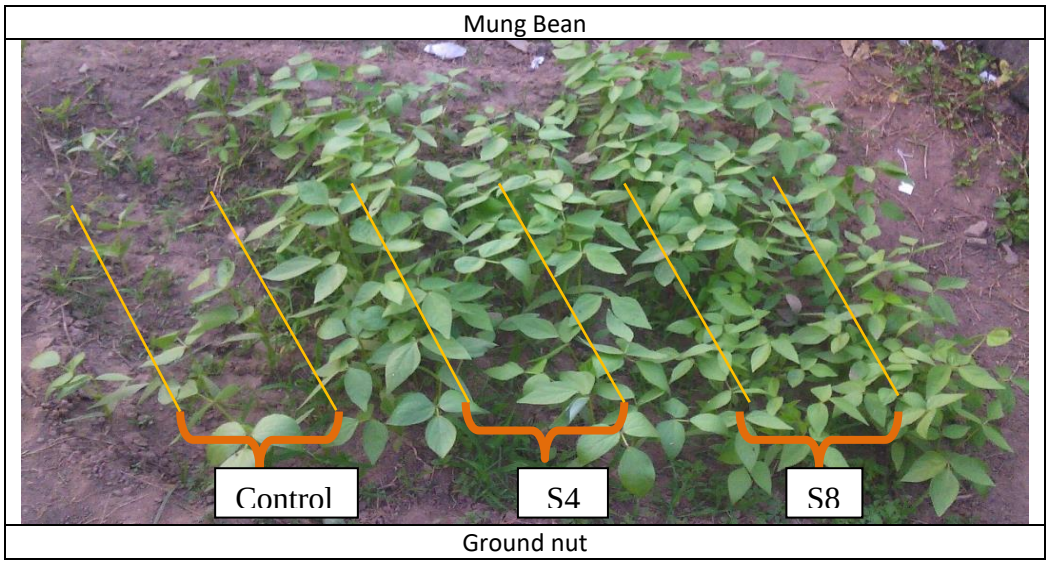
Isolate code	O.C (g/kg)	N (kg/ha)	P_2O_5 (kg/ha)	K_2O (kg/ha)	pH	EC (ds/m)
Control	0.181 ±0.01[a]	147.6±0.29[a]	315.6 ±2.24[a]	43.4±0.26[a]	6.0±0.03[a]	0.066±0.01[a]
S4	0.184±0.01[b]	148.1±0.12[a]	321.3±0.81[b]	44.7±0.12[b]	6.2±0.05[a]	0.068±0.01[b]
S8	0.188±0.01[c]	150.0±0.08[b]	324.0±0.97[c]	48.8±0.06[c]	6.3±0.03 [b]	0.068±0.01[b]

*Values are the mean ± SEM and differ significantly as per DMRT by LSD (P <0.05). Mean values in each column with same superscript (s) do not differ significantly as per DMRT.

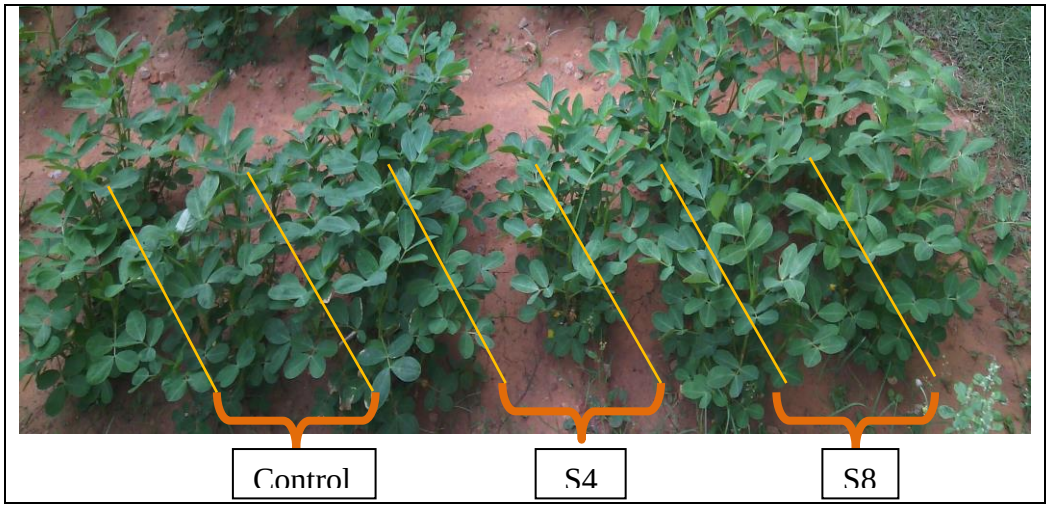
Field Trial

Biometric observation of Mung Bean increases with treatment of *Bacillus* sp. S8 compare to control and *Bacillus* sp. S4. Root and Shoot length, Dry and Wet weights were found to be excellent in *Bacillus* sp. S8 treatment compare to *Bacillus* sp. S4 and control. The statistical approach also revealed significant effect of Plant growth in natural environmental condition (P <0.05). In present study, the potential bacterial isolates S4 and S8 were assessed for the growth promotion and yield of Mung bean and Groundnut under field conditions. Results revealed that there was significant increase in plant growth, Root length, Shoot length, Dry weight, Wet weight were of Mung Bean and Groundnut with the inoculation of selected PGPR strains. This results of present study clearly showed the efficiency of *Bacillus* sp. S8 in plant growth enhancement, phosphorus uptake and soil fertility (Figure 2 and 5). Many studies in relation to crop improvement by PGPR were carried out either in pot cultures or field conditions [3, 16].

Figure 5: Growth of Mung Bean and Ground nut in Field condition



ISSN: 0975-8585



CONCLUSION

From this experimental study we have established PHAs accumulating PGPR isolates not only show in-vitro activity but they are showing excellent growth of plants in natural environmental condition. Among all *Bacillus* species *Bacillus* sp. S8 exhibited better activities, which can be directed for farming. However, a lot of research is needed for evaluating the biotechnological properties of these PHAs accumulating Bacillus species showing PGPR activity.

Conflict of interests: Declared None

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