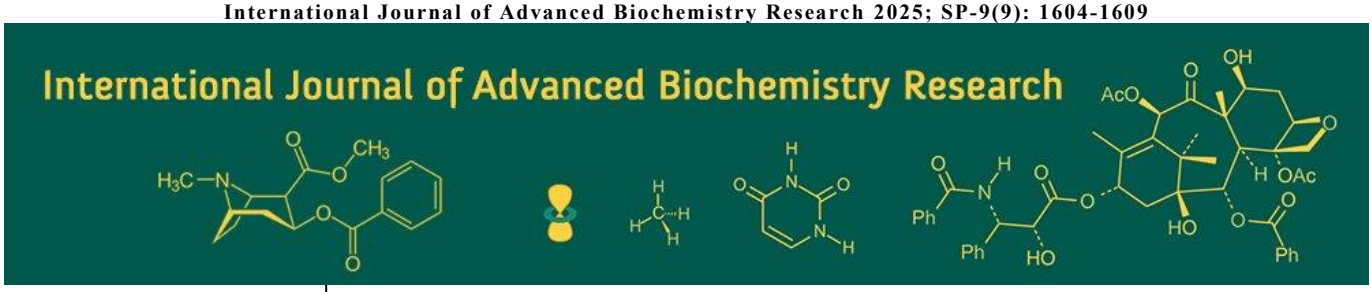

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Effect of bio-inoculants (*Azotobacter* and PSB) on *r*hizosphere microbial and enzymatic status in Chilli (*Capsicum annuum* L.) crop

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Abstract
The study was conducted to characterize Azotobacter and PSB and assess the impact of bio-inoculants on the rhizosphere microbial and enzymatic status of chillies. The experiment was conducted with seven treatment and four replications. *Azotobacter* isolates (Azoto-122, Azoto-146 and Azoto-137) in Jensen's medium produced circular, gummy, convex, with entire margin, whitish in colour while PSB (PSB-126) isolated in Pikovskaya's agar media produced creamy to yellowish round with entire margin and, showing moderate clear zone surrounding the colony growth. Azoto-137 isolate showed positive reaction for starch hydrolysis, while positive reaction for catalase test, urease test, and oxidase test was observed for all isolates. Glucose fermentation and Gas evolution occurred with PSB isolate and in *Azotobacter* strain (Azoto-137). Analysis of rhizosphere soil of pot grown chilli crops, showed significant increase in the *Azotobacter* population from (8.64 $\log_{10}$ cfu g^{-1} soil) to (8.79 $\log_{10}$ cfu g^{-1} soil), PSB population from (6.09 $\log_{10}$ cfu g^{-1} soil) to (7.54 $\log_{10}$ cfu g^{-1} soil) also improved dehydrogenase activity from (2.86 µg TPF g^{-1} soil h^{-1}) to (12.49 µg TPF g^{-1} soil h^{-1}), phosphatase activity from (2.23 µg PNP g^{-1} soil hr^{-1}) to (15.11 µg PNP g^{-1} soil hr^{-1}), and basal soil respiration rate from (0.55 mg CO2 h^{-1} $100g^{-1}$) to (1.07 mg CO2 h^{-1} $100g^{-1}$) were recorded under the treatment T_7 (Azoto-137+PSB-126) as compare to control. Thus, the dual application of bio-inoculants *Azotobacter* and PSB showed significant best result and in the treatment T_7 (Azoto-137+PSB-126) recorded significant highest plant growth and rhizosphere soil analysis of chilli.

Keywords: *Azotobacter*, PSB, chilli, rhizosphere, bio-inoculants, and microbiology

Introduction
Biofertilizers are microorganisms that enrich the soil by enhancing the availability of nutrients for crops. In short, Biofertilizers are the replacement of chemical fertilizers (Bhattacharya and Mishra) [4]. They are economically and ecologically better than chemical fertilizers at improving the quality and productivity of crops by producing plant growth-promoting hormones and phytoalexins. They also reduce the application for chemical fertilizers, ensuring environmental safety. The most commonly used Biofertilizers in horticulture crops are *Azotobacter* (Afzal and Bano) [1], *Bacillus* (Orhan *et al*.,) [15], *Azospirillum*, and *Pseudomonas* (Naiman *et al*.,) [12].
Azotobacter is a free-living, aerobic, nitrogen-fixing, non-symbiotic heterotrophic bacteria found in soil. They can fix an average of 20 kg N^{-1} ha^{-1} year. *Azotobacter* also produces biologically active compounds such as phytohormones like auxins, IAA, and GA (Ahmad *et al*.) [2], they also produce Siderophores and antifungal substances, thereby stimulating plant growth (Oblisami *et al*.) [14]. Biofertilizers *Azotobacter* is known to fix a substantial amount of atmospheric nitrogen (Ghoname and Shafeek) [8].
Phosphate solubilizing bacteria (PSB) are important biofertilizer that enhance the availability of phosphorus (P) to plants by converting insoluble forms of phosphorus into soluble forms that plant can absorb. Currently, PSB has attracted the attention of agriculturists as soil inoculums to improve plant growth and yield. Several studies have shown that *Pseudomonas fluorescens* as a soil bioinoculants improves the growth of plants by various mechanisms, like the production of antifungal compounds, siderophore production, plant hormone production and phosphate solubilization. Strains from the genera *Pseudomonas*, *Bacillus* and *Rhizobium* are among the most powerful phosphate solubilizers (Rodriguez) [16].

Phosphate solubilizing bacteria (PSB) and Vasicular arbuscular mycorrhizae (VAM) make more available phosphate in soil, which is supplied to the crop, enhance fertilizer use efficiency, soil fertility status, and ensure partial savings of fertilizers, which in turn reduce the cost of cultivation.

The present investigation, we tried to study developing an efficient nutrient management system involving biofertilizers i.e., using beneficial *Azotobacter* and PSB strains to maintain soil fertility and for better crop production.

Material and Methods

An experiment was conducted that included rhizosphere microbial soil analysis of the chilli crop as well as chemical and morphological characterization of the microorganisms Azotobacter and PSB (pseudomonas). The experiment was carried out at Department of Agricultural Microbiology, college of Agriculture Raipur with seven treatments and four replications. The treatment details are T_1 (Control), T_2 (Azoto-122), T_3 (Azoto-146), T_4 (Azoto-137), T_5 (Azoto-122+PSB-126), T_6 (Azoto-146 + PSB-126), T_7 (Azoto-137 + PSB-126). The *Azotobacter* and PSB isolates were obtained from departmental culture collection bank of Department of Agricultural Microbiology, for this investigation. The isolates of Azotobacter and PSB (pseudomonas) were characterized in a laboratory setting using Gram staining, colony morphology features, starch hydrolysis, catalase, urease, oxidase, TSI (triple sugar iron) test and antibiotics. Gram staining, colony morphology and colony characters were performed on cultures that were 48 hours to 7 days old. On agar medium, the colony characteristics *viz*. margin, elevation, size and colour were noted. Further appropriate dilution i e. 1.0 ml of *Azotobacter* and PSB was transferred into the Petri plates containing Jensen's media and Pikovskaya's media respectively. For 2-3 days were spent incubating the plates at 28 ± 2°C. In the gram's staining a thin and smooth bacterial smear was made on glass slides. To which we, apply crystal violet followed by washing of slide with distilled water. Gram's iodine solution was applied to each slide then to remove the iodine solution; these slides were washed in 95 percent ethyl alcohol. The slides were cleaned and drained with distilled water once more. The slides were then poured with Safranin red for 30 seconds. They were then rinsed in distilled water. Gram positive cells were looked purple, while Gram negative cells were looked pink or red (Aneja) [3].

The isolates underwent a variety of biochemical tests, including the Starch hydrolysis test, Catalase test, Urease test, Oxidase test, TSI Test (triple sugar iron test) and antibiotic teat. The enzyme amylase, which breaks down starch is detected by the starch hydrolysis method. Amylase converts starch, a polysaccharide, to maltose, a disaccharide and some monosaccharide such as glucose. Isolates were streaked singly on starch agar media and incubated at 28± 2ºC for 48 hours. After that, the plates were flooded with iodine solution to test the isolates starch digestion capacity. A yellow zone around the colony indicated starch hydrolysis, while a blue/black area indicated starch presence. For catalase test a bacterial colony was transferred on the surface of a clean, dry glass slide using a loop or sterilized wooden stick. A drop of 3% H_2O_2 was dropped on the slide, and the combination of quick oxygen evolution (within 5-10 seconds) as proven by bubbling indicated a positive result, whereas no bubbles or simply a few scattered bubbles indicated a negative result. In Urease test *Azotobacter* and PSB cultures were added to the Urea agar slants. At 37°C for 24 hours, the slants were incubated. If the bacterial growth zone on the orange-colored urease slant turns pink, urease hydrolysis activity is confirmed. The oxidase test is used to see if the isolates can make cytochrome oxidase. *Azotobacter* and PSB isolates were inoculated to nutrient agar medium plates which were inoculated for 24-48 hours at 37°C. After that a commercially available oxidase disc containing the reagent is placed on the plates and waited for 10 seconds to see the colour to change. Deep purple colour change was deemed positive, while a change later than 10 seconds or no colour change was considered negative.

Triple Sugar iron test is used for the separation of microorganisms by the virtue of their ability to determine the use of carbohydrate fermentation and hydrogen sulphide production. The microorganisms that ferment glucose change the medium's colour from red to yellow and produce a range of acids. The Triple Sugar Iron agar medium was set in the sloped form with a butt about 1inch long slants were stabbed, incubated at 28±2ºC for 48 hours and results were observed there after (Krumwiede and Kohn) [10]. The presence of alkaline (red) slant and an acid (yellow) butt after incubation indicates that microorganisms is a glucose fermentation but are unable to ferment lactose or sucrose (or both), in addition to glucose, in large amounts acids do produce. Gas production (CO_2) is seen through the presence of cracks or bubbles in the medium, when the accumulated gas is saved. The antibiotic resistance of the strain was assayed with the help of agar diffusion method using filter paper disc impregnate with the test antibiotic in different concentration (Date and hurse) [5]. The actively growing culture of the test *Azotobacter* and PSB isolates were prepared in Jensen's and Pikovskaya's broth media. Sterilized petri-plates containing solidified medium were surface inoculated with 0.5ml of actively growing culture of the test strain and spread uniformly by means of a sterilized spreader

Application of Biofertilizers, Seed treatment and Seed sowing

Liquid biofertilizers were given to chilli crops in pots according to treatment description through soil as well as seed treatment methods. Liquid biofertilizers of *Azotobacter* and PSB isolates separately applied to soil in the pot before sowing @ 5 ml pot^{-1}; About 24 hours before sowing, followed by light irrigation. Seeds of chilli were treated with all *Azotobacter* and PSB isolates, for inoculation, matured broth of *Azotobacter* and PSB isolates was diluted with sterilized aqueous so that 1:4 dilution was attained. In this diluted broth sugar was added @ 0.5g $100ml^{-1}$ broth as a sticking agent. Four holes (2 to 3 cm deep) per pot were made with the help of sterilized glass rod maintaining equal distance from hole to hole. Sowing of 4 seeds was done by placing one seed in each hole with the help of sterilized forceps.

Soil microbial analysis

Soil microbial analysis *viz*, estimation of microbial population, dehydrogenase test in soil, phosphatase activity test and basal soil respiration study were conducted in microbiology laboratory. Rhizosphere soil samples from

different treatments were collected for enumeration of *Azotobacter* and PSB. The plating and serial dilution methods were used for enumeration (Subba Rao) [18]. Following colony counting, the populations were converted to cfu g^{-1} of dry soil using the formula (Schmidt and Caldwell) [17].

Dehydrogenase activity was determined with adding 0.2 ml of 3% Triphenyl Tetrazolium Chloride solution was added in each of the tubes to saturate the soil. The tubes were incubated for 24 hrs at 37ºC and then 10 ml of methanol was added to them. The tubes were shaken and left to stand for 6 hrs. Clear pink coloured supernatant was withdrawn and readings were taken with a colorimeter. (Klein *et al*.) [9]. The acid phosphatase activity was calculated with the experimental soil of the chilli pots' pH of 6.9 in mind. Out of two sets one was used as control. 4 ml of modified universal buffer and 0.2 ml of toluene were added. To one set of samples, 1 ml of p-nitrophenyl phosphate solution was added. After that, the flasks were incubated for an hour at 37ºC. Four ml of 0.5 M NaOH and one ml of 0.5 M $CaCl_2$ were added following incubation. The remaining set (control) of samples received 1 ml of p-nitrophenyl phosphate. The yellow colour intensity of the filtrate was measured with a blue filter. The result obtained using the MUB (Modified Universal Buffer) of pH 6.5 was the measure of acid phosphatase (Evazi and Tabatabai) [9]. The basal respiration of soil was determined by measuring the rates of CO_2 evolution. One litter conical flask was filled with 100 g of oven-dried soil. 20 ml of 0.5N NaOH were placed in test tubes. Rubber stoppers were used to keep the flasks airtight while the tubes were hung inside the conical flasks using thread. The flasks were incubated at 28°C for about 20 hrs. The NaOH solution was promptly moved from the test tube to a 150 ml conical flask following incubation. A few drops of phenolphthalein indicator were added to 5 ml of 3N $BaCl_2$ solution. Titrate the material gradually with regular 0.5N H_2SO_4 until the pink hue simply goes away.

Results and Discussion

Retrieval of different strains of *Azotobacter* and PSB isolates

Azotobacter and PSB isolates cultures were revived by inoculating *Azotobacter* in Jensen's medium and PSB in Pikovskaya's medium of pH (7.0) and temperature (28±2ºC) each. After two days of incubation, bacterial colonies were obtained, and pure cultures were preserved as slant in the refrigerator. Active culture broths of isolates were prepared and subjected to morphological and biochemical characteristics.

Colony morphology and Gram staining

The *Azotobacter* isolates produced translucent, nearly round, and gummy colonies which varied in size between 1.5 to 2.0 mm and were whitish in colour (Table-1). *Azotobacter* colony morphological characteristics were examined which revealed. After the gram-staining, the bacteria assumed a red colour, which indicated that it was a Gram (-ve) stain. Phosphorous solubulizing bacterial isolates were found capable of forming a clear zone on solid Pikovskaya's media. These were further studied for colony morphology, Gram staining. The colonies of all the isolates were found to be round and yellowish in colour. All the isolates were gram-negative. Based on the biochemical and morphological tests, the isolated bacteria were identified in consultation with Bergey's Manual of Systematic Bacteriology. PSB isolate was found to belong to *Pseudomonas* sp. The identification studies are also in coordination with the works done by Gadagi and Sa [7] and El-Dsouky *et al*. [6].

Biochemical characterization of isolates

Azotobacter strain, Azoto-137 was recorded as starch hydrolysis positive, and other *Azotobacter* strains (Azoto-122 and Azoto-146,) were recorded as starch hydrolysis negative. Also isolate, PSB-126 showed negative test for starch hydrolysis test. All strains of *Azotobacter* (Azoto-122, 137, and 146) and PSB-126 showed positive results for Catalase test, Urease test and Oxidase test. In TSI (triple sugar iron) test *Azotobacter* strain, Azoto-137 showed positive result for Glucose fermentation and negative result for Gas and H_2S production, while other *Azotobacter* strains (Azoto-122 and Azoto-146) showed no Glucose, Lactose and Sucrose fermentation as well as no Gas and H_2S production. Whereas, PSB-126 showed positive result for Glucose fermentation and Gas evolution, (Table-2).

Antibiotic susceptibility test, the susceptibility/resistance of *Azotobacter* and PSB isolates against some antibiotics i.e., Tetracycline (10 µg), Chloramphenicol (10 µg), Kanamycin (5 µg) and Streptomycin (25 µg) was determined and the results were indicated in Table-3.

Effect of bio-inoculants on chilli

Effect of bio-inoculants (*Azotobacter* and PSB isolates) were evaluated on overall the growth performance of chilli in pot experiment. The dual application of bio-inoculants (Azoto-137+PSB-126) on chilli crop showed significant plant growth and remained at per with (Azoto-146+PSB-126). Superior effect on chilli showed in both treatment over rest of treatments. Significantly highest plant height was recorded in T_7 followed by in T_6 and in T_5 at 90 days however $T_7$and T_6 were at per. Data on plant height recorded at five different stages of (30, 45, 60,75 and 90 DAS) chilli crop growth showed in Fig 1.

Rhizosphere soil microbial analysis of chilli

Fig 2 presents data on the populations of Azotobacter and phosphate-solubilizing bacteria (PSB) per gram of soil in the rhizosphere of chili plants, as influenced by various treatment effects during the flowering stage. *Azotobacter* population density at the flowering stage increased significantly over control under different treatments. The treatment T_7 (Azoto-137+PSB-126) recorded the maximum *Azotobacter* population (8.79 $\log_{10}$ cfu g^{-1} soil), followed by T6 (8.778 $\log_{10}$ cfu g^{-1} soil). The minimum *Azotobacter* population (8.64 $\log_{10}$ cfu g^{-1} soil) was recorded in treatment T_1 (control). PSB population density at the flowering stage increased significantly over control. The treatment T_7 (Azoto-137+PSB-126) recorded the maximum PSB population (7.54 $\log_{10}$ cfu g^{-1} soil), followed by treatment T6 (7.47 $\log_{10}$ cfu g^{-1} soil). The minimum PSB population (6.09 $\log_{10}$ cfu g^{-1} soil) was recorded in treatment T_1 (control).

Dehydrogenase activity

Table-4 provided information on how various treatments affected the dehydrogenase activity (DHA) in the rhizosphere soils of chilli crop pots. The treatments with the highest dehydrogenase activity at harvesting time were T_7 (Azoto-137+PSB-126), T_6, T_5, T_4, T_3, T_2, and T_1 (Control),

with T_7 having the highest activity (12.49 µg TPF g^{-1} soil h^{-1}). Above observations were in close agreement with Nowark [13] and Wyszkowska and Kucharski [19]. Who claimed that dehydrogenase activity is a reflection of the biological status of soil.

Phosphatase test

The result on Acid phosphatase activity (µg PNP g^{-1} soil hr^{-1}) in rhizosphere soil of pot grown chilli crop after harvesting were presented in Table-4. were significant. After harvest treatment T_7 (Azoto-137+PSB-126) was recorded significantly higher acid phosphatase activity (15.11 µg PNP g^{-1} soil hr^{-1}) followed by T_6 (13.44 µg PNP g^{-1} soil hr^{-1}). The lower acid phosphatase activity was recorded in the treatment T_1 (Control) (2.23 µg PNP g^{-1} soil hr^{-1}). Similar findings were reported by Linu *et al*. [11].

Basal soil respiration test

Basal soil respiration rate (BSR) after harvest the treatment T_7 (Azoto-137+PSB-126) was recorded highest Basal soil respiration (1.07) followed by treatment T_3 (0.97. The minimum Basal soil respiration was recorded in treatment T_1 (Control) (0.55) presented in Table 4.

Table 1: Cultural and Morphological characteristics of *Azotobacter* and PSB isolates used as bio-inoculants in experiment.

Isolates	Colony morphology	Gram staining
***Azotobacter* isolates in Jensen's medium**		
Azoto-122	Gummy, round and convex, entire margin, whitish in colour	Gram –ve
Azoto-137	Gummy, round and convex, entire margin, whitish in colour	Gram –ve
Azoto-146	Gummy, round and convex, entire margin, whitish in colour	Gram –ve
PSB isolates in Pikovskaya's medium		
PSB-126	Smooth round, entire margin, showing clearing zone, yellowish in colour	Gram –ve

Table 2: Biochemical characterization of isolates used as bio-inoculants in experiment

Biochemical test	Azoto-122	Azoto-137	Azoto-146	PSB-126
Starch hydrolysis	-ve	+ve	-ve	-ve
Catalase test	+ve	+ve	+ve	+ve
Urease test	+ve	+ve	+ve	+ve
Oxidase test	+ve	+ve	+ve	+ve

Triple Sugar Iron Agar Test (TSI test)					
Isolates	**Glucose fermentation**	**Lactose fermentation**	**Sucrose fermentation**	**Gas production**	**H_2S production**
Azoto-122	-ve	-ve	-ve	-ve	-ve
Azoto-137	+ve	-ve	-ve	-ve	-ve
Azoto-146	-ve	-ve	-ve	-ve	-ve
PSB-126	+ve	-ve	-ve	+ve	-ve

Table 3: Determination of Antibiotic susceptibility of different bacterial isolates

S.N.	Isolate No.	Antibiotics							
		Tetracycline (10 µg)		Chloramphenicol (10 µg)		Kanamycin (5 µg)		Streptomycin (25µg)	
		Sensitivity	Zone dia. (mm)	Sensitivity	Zone dia. (mm)	Sensitivity	Zone dia. (mm)	Sensitivity	Zone dia. (mm)
1	Azoto-122	S	21	S	10mm	S	12mm	S	28mm
2	Azoto-137	S	15	R	0mm	S	12mm	S	30mm
3	Azoto-146	S	22	S	10mm	S	19mm	S	30mm
4	PSB-126	S	21	R	0mm	S	10mm	S	13mm

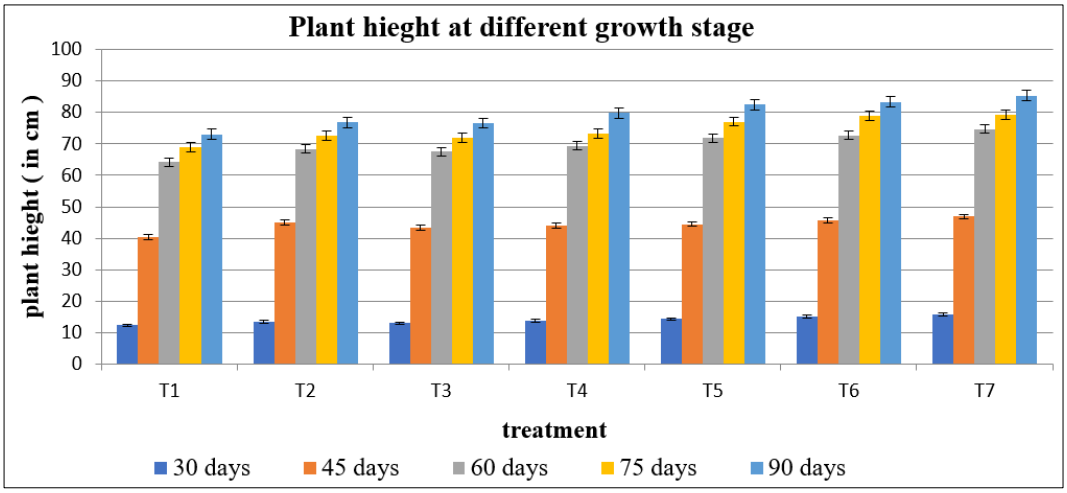

Fig 1: Effect of Bio-inoculants on plant height of chilli at different growth stages

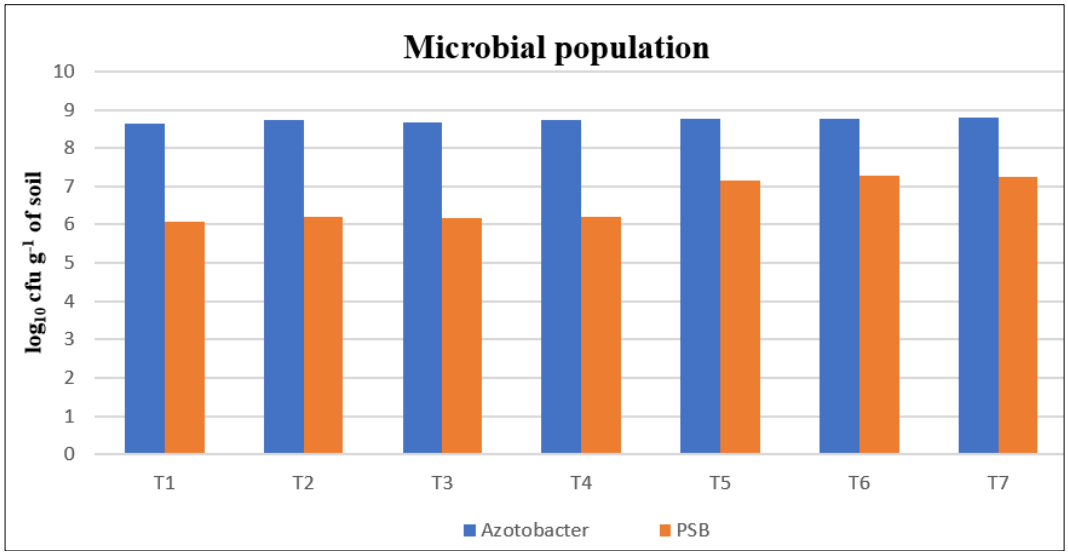

Fig 2: Effect of Bio-inoculants on microbial population of chilli crop at flowering stages

Table 4: Effect of bio-inoculants on dehydrogenase activity, phosphatase test and basal soil respiration rate on rhizosphere soil in pot-grown chilli after harvest.

Treatment	Treatment detail	Dehydrogenase activity of composite rhizosphere soil sample (µg TPF g^{-1} soil h^{-1})	Phosphatase activity (µg p-NP g^{-1} soil h^{-1})	Basal soil respiration rate (mg CO_2 /h/100g)
T_1	Control	2.86	2.23	0.55
T_2	Azoto-122	8.86	7.74	0.95
T_3	Azoto-146	5.96	10.35	0.97
T_4	Azoto-137	6.88	14.88	0.80
T_5	Azoto-122+PSB-126	10.73	12.29	0.93
T_6	Azoto-146+PSB-126	7.74	13.44	0.73
T_7	Azoto-137+PSB-126	12.49	15.11	1.07
	SEm±	0.85	0.406	0.034
	CD (0.05%)	2.55	1.196	0.100

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