Int.J.Curr.Microbiol.App.Sci (2019) *8*(5): 2058-2068

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 8 Number 05 (2019)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2019.805.239

Characterization and Screening of Native Isolates of PSB and *Azotobacter* under *in vitro* Conditions

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Keywords

PSB, *Azotobacter*, BNF, Phosphorus solubilizing capacity.

Article Info

Accepted:
17 April 2019
Available Online:
10 May 2019

A B S T R A C T

The present study was conducted at Department of Agriculture Microbiology, College of Agriculture, IGKV, Raipur, C.G. during the year 2018-19 to characterize and screen different native isolates of PSB and *Azotobacter*. 14 microbial isolates were biochemically characterized and screened under in-vitro conditions for their plant growth promoting properties. Among 14 tested isolates Azoto-B-44, Azoto-146, Azoto-B-126, PSB-S-88, PSB-H-27, PSB-S-170, PSB-S-71, PSB-H-5, PSB-S-162 were shown positive results for TSI and Citrate test. PSB-172 and PSB-S-64 were shown positive for MR test and PSB-S-88, PSB-S-71, PSB-H-5, PSB-S-165 and PSB-S-162 were found positive for Gelatin liquefaction test. Rest of all isolates was negative towards above tests. All the isolates were taken for their antibiotic susceptibility study. Some isolates were found susceptible for Tetracycline (30mcg) and streptomycin (10mcg). In N-fixation study of *Azotobacter* isolates Azoto-B-126 found higher N-fixer, it fixed 3.25mg N/gm of sucrose. Azoto-123, Azoto-146 and Azoto-B-126 found significantly superior and at par for N-fixing capacity over Azoto-B-44. Ten isolates of PSB were screened for their P-solubilizing capacity. PSB-H-5 was found highest P-solubilizer (894.51 µg/ml), however all the isolates found significantly superior for P-solubilizing capacity over control. All the PSB isolates were also tested for their solubilizing efficiency of phosphorus in the form of solubilization zone. PSB-H-27 was found highest solubilization efficiency with solubilization zone of 14 mm diameter, however it found at par with PSB-S-162, PSB-S-165, PSB-S-71, PSB-H-5 and PSB-S-170.

Introduction

Bio-fertilizers are the bio-inoculants of specific beneficial microorganisms that promote the growth and development of plant crops by converting the unavailable form of nutrients into available form. These biofertilizers also improve the soil fertility (Sivasakthivelan and Saranraj, 2013). Biofertilizer contains living microorganisms which promote plant growth mainly by increasing the availability of primary nutrients (nitrogen and phosphorus) to the host plant. Organisms that are commonly used as biofertilizers component are nitrogen fixer, potassium and phosphorus solubulizer or with

the combination of molds or fungi. They are the best alternative to the chemical fertilizers (Naz and Bano, 2010).

Azotobacter, a free living heterotrophic nitrogen fixing bacterium, belongs to the family Azotobacteriaceae (Becking, 1981). *Azotobacter* species are found in soil, water, rhizosphere etc. It is a gram-negative motile soil organism and can be isolated and cultured ex-situ conveniently. *Azotobacter* is a highly aerobic organism, which fixes atmospheric nitrogen asymbiotically (Tejera *et al.,* 2005). Besides, nitrogen fixation, *Azotobacter* also produces growth hormones viz., auxin, cytokinin, thiamine, riboflavin, nicotine, indole acetic acid and gibberellins, thereby stimulating plant growth. Phosphorus is an essential element for plant development and growth. Plants acquire P from soil solution as phosphate anions. There are various types of soil microbes which can solubilize this fixed form of P and make it available to plants (Richardson, 2001). Application of phosphorus along with phosphate solubilizing bacteria (PSB) improve P uptake by plants and yield indicating that the PSB are able to solubilize phosphates and to mobilize phosphorus in crop plants.

The plant growth benefits due to the addition of PSB include increases in germination rate, root growth, yield, leaf area, chlorophyll content, tolerance to drought, shoot and root weight (Abbasi *et al.,* 2015).

Biofertilizers are more effective in soil when sufficient population of effective microbes is used to prepare them. Screening of microbes is necessary to select the effective crop beneficial microbe(s).

Screening allows the discarding of many valueless microorganisms, at the same time it allows the easy detection of the most effective microorganisms (Sagervanshi *et al*., 2012). Identification and characterization of microorganisms is a key part of the microbial management. This technique is useful to identify bacteria or other unknown microorganisms in the bacterial population. The aim of this study is to revive *Azotobacter* and PSB isolates of microbial repository of Dept. of Agril. Microbiology, College of Agriculture, Raipur, characterize them and through systematic screening select the best performing *Azotobacter* and PSB isolates for their further use in crop production.

Materials and Methods

Collection of bacterial samples

Bacterial samples were collected from Microbiology repository of Department of Agricultural Microbiology, College of Agriculture, Raipur, C.G.

Sub-culturing of isolates

Sub-culturing of phosphate solubilizing bacteria and *Azotobacter*, Pikovskaya's media and Jensen's agar media were used, respectively. The isolates were revived by inoculating them in respective broths and incubated for 72 hrs. After incubation, the containing PSB and *Azotobacter* isolates was streaked on respective media plates and incubated them at 28±2°C for 48 hours. Pure colonies were selected and transferred them to respective agar slants and preserved them at 4°C for further study.

Study of phenotypic and biochemical properties of the collected isolates

Pure cultures of the collected isolates were characterized using criteria of Bergey's Manual of Systematic Bacteriology (Brenner *et al.,* 2005). The Following morphological and biochemical tests were used.

Phenotypic characterization

The Pure isolates were tested for the following morphological properties in which different shapes, size, elevation, colony size; Colony pigmentation, Gram reaction and shape of cell were examined.

Biochemical characterization

The isolates were characterized using standard biochemical methods as given in Bergey's Manual of Systematic Bacteriology (2001). The Catalase test, MR-VP test, citrate test, urease test, Indole production test and gelatin liquefaction test were carried out. A very specialized test the Triple Sugar Iron Agar test was conducted in order to diagnose them for glucose, lactose, and sucrose fermentation along with peptone catabolization, gas and H_2S production ability (Blazevic and Ederer, 1975).

In vitro screening of *Azotobacter* isolates for their nitrogen fixing ability and PSB for their P-solubilizing capacity

Nitrogen fixing ability: nitrogen estimation by microkjeldhal method

The amount of nitrogen fixed by *Azotobacter* isolates was estimated by Microkjeldhal method given by Jackson (1967). The collected *Azotobacter* cultures were inoculated to 5ml of N free broth medium. It was inoculated for 48 hours. 1 ml of this broth was inoculated to 50 ml N free broth medium and inoculated for 15 days. 20ml of this culture was used for nitrogen estimation by following the standard procedure of Microkjeldhal technique (Reis *et al.,* 1994).

Phosphorus solubilizing capacity

The flask containing 50ml of aliquots of inoculated cultures of Pikovskaya's broth medium were filtered through Whatman No. 1 paper to remove insoluble phosphate and centrifuged at 10,000 rpm for 10-15 minutes. After centrifugation, 10 ml aliquot was taken and 10 ml of Barton's reagent was added and the volume made up to 50 ml. After 10 minutes, the resultant colour was read in a spectrophotometer using 420 nm wavelength (Koening and Johnson, 1942).

Phosphate solubilization efficiency

Sterilized Pikovskaya's media was poured into sterilized Petri plates, after solidification of the media; a pin point inoculation of the Petri plates was made on plates under aseptic conditions. The plates were incubated at 28°C for 7-10 days. Then the ability of PSM to solubilize the insoluble phosphate was studied by the determination of solubilization efficiency (SE).

Where,

$$SE = \frac{\text{Solubilization diameter}}{\text{Growth diameter}} \times 100$$

Antibiotic study

Antibacterial activity was carried out using disc diffusion method. The tests were conducted with 4 different antibiotic disc (Streptomycin-10mcg/disc, Tetracyclin-30mcg/disc, Penicillin-G-10mcg/disc, and Ampicillin-10mcg/disc). Antibiotic disc were placed at the center of the broth culture plates and incubated at 28±2°C for 3-4 days. Antibiotic sensitivity was observed by measuring the hollow zone diameter of the studied organism.

Results and Discussion

The present research was conducted for characterizations of14 isolates which were obtained from the microbial repository of

dept. of Agril. Microbiology, IGKV, Raipur and the isolates were screened for nitrogen fixing capacity and phosphate solubilizing capacity.

Phenotypic characterization of selected isolates

All the 14 isolates were selected for further phenotypic studies and were confirmed as *Azotobacter* and Phosphorus solubilizing *Bacillus* and *Pseudomonas sp*. based on morphological characteristics and their gram staining behaviour (Table 1 and Figure 1). Karpagam and Nagalakshmi (2014) also isolated bacteria from agriculture soils and reported the genera *Bacillus, Pseudomonas* and *Azotobacter* as PSB.

Biochemical test

A series of biochemical tests were carried out for a better understanding of the physiochemical functions going on within the cell. In this study, among different *Azotobacter* isolates Azoto-B-44, Azoto-146 and Azoto-B-126 shown positive results for TSI test and Citrate test. Similarly, among different PSB isolates PSB-S-88, PSB-H-27, PSB-S-170, PSB-S-71, PSB-H-5 and PSB-S-162 shown positive results for TSI and Citrate tests.PSB-172 and PSB-S-64 found positive for MR test and PSB-S-88, PSB-S-71, PSB-H-5, PSB-S-165 and PSB-S-162 found positive for Gelatin liquefaction test. Rest of all PSB isolates found negative in rest of all tests (Table 2 and Figure 2).

In vitro screening of *Azotobacter* and PSB isolates

Nitrogen fixing ability

The nitrogen fixing ability of 4 local *Azotobacter* isolates was tested for initial screening of the isolates. Statistically highest nitrogen fixing ability was observed in Azoto-B-126 (3.250 mg N/gm of sucrose) followed by Azoto-146 which fixed 2.750 mg N/gm of sucrose after fifteen days of incubation. Azoto-123, Azoto-146 and Azoto-B-126 found significantly superior and at par for N-fixing capacity over Azoto-B-44. Similar findings were also reported by Bag *et al.*, (2017) that nitrogen fixing capacity of *Azotobacter* under *in vitro* condition, ranges between 3.16 – 12.66 mg N/gm of sucrose. Similarly Gupta *et al.*, (1992) showed that *Azotobacter* can fix atmospheric nitrogen at 1.47 to 1.50 (Average, 1.49) mg N per gm of carbon source, whereas, Gondotra *et al.*, (1998) found the range as 13.3 to 21.6 mg N/g glucose (Table 3).

Phosphorus solubilizing capacity

All 10 isolates were screened for their potential to solubilize the phosphate. All isolates showed much or less variations in their potential for phosphate solubilization ranging from 263.72µg P/ml to 894.51µg P/ml as per result recorded in table 4 and presented by bar diagram in figure. The isolates PSB-H-5 showed the highest phosphate solubilizing capacity i.e. 894.51 µg P /ml followed by PSB-H-27 and PSB-S-71 which solubilized 803.92 µg P/ml and 768.08 µg P/ml, respectively, whereas the isolate PSB-S-64 (263.72 µg P /ml) was showing the least potential. All the PSB isolates found significantly higher of P-solubilizing capacity over control (Table 4). Sharon *et al.*, (2016) also reported that PSB isolates have the similar character of P-solubilization activity ranges between 328 mg P/L to 956 mg P/ L.

Phosphate solubilization efficiency

All the PSB isolates were examined for their ability to solubilize phosphate sources on agar media supplemented with tri-calcium phosphate. These isolates formed a clear zone

diameter of between 10-14mm and the largest clear zone diameter of 14 mm was recorded in PSB-H-27 and the least clear zone was obtained in PSB-172 and PSB-S-64 i.e. 10mm. Similarly, highest solubilization efficiency was recorded in PSB-H-27 which was 100 percent (Table 5 and Figure 3). Similar results were found by Selvi *et al.*, (2017) that Phosphate solubilizing bacteria were able to produce 0.2 cm to 1.0 cm of solubilization zone. Solubilization efficiency (SE) varied from 13.04 percent to 85.71 percent on 7 days of incubation period.

Antibiotic sensitivity of isolates

On the basis of the pattern of antibiotic response, all the bacterial isolates were distinguishable from each other. Azoto-B-126, Azoto-123, Azoto-146, PSB-H-5, PSB-S-170, PSB-H-27 and PSB-S-71 observed sensitive towards Tetracycline (30mcg/disc) with the inhibition zone of diameter 24, 11, 17, 37, 36, 38 and 34 mm, respectively. Whereas, Azoto-B-126, Azoto-B-44 and Azoto-146 found sensitive towards Streptomycin (10mcg/disc) with the inhibition zone of diameter 19, 23 and 18 mm respectively (Table 6 and Figure 4). Similar results found by Gupta *et al.,* (2005) that isolate L-11 and L-20 showed resistance to antibiotic discs of Kenamycin (30µg), Gentamycin (30µg) and Ampicillin discs (10µg). Promising rhizobial isolate L-4 showed maximum zone of inhibition (zone dia. 25.3 mm) with Kenamycin (30 mcg) and with Gentamycin (30 mcg). Isolate L-3 showed maximum susceptibility (zone dia. 23.0 mm) might be due to more fusaric acid production (Singh and Saxena, 2002).

Kumar and Raghuram (2014) also recorded that solubilization of phosphorus with zone of range 12-18 mm was recorded in *Azotobacter* and PSB isolates and maximum solubilization efficiency of 125% was recorded while it is between 40-75% in rest of all strains.

Table.1 Morphological characteristics and gram staining behaviour of isolates

S.No.	Microorganisms	Colony morphology				Gram staining
	Isolate No.	Colour	Forms	Margin	Elevation	Gram reaction
1.	*Azoto*-123*	White	Circular	Entire	Convex	_
2.	*Azoto*-B-44*	White	Circular	Entire	Convex	_
3.	*Azoto*-146*	White	Circular	Entire	Convex	_
4.	*Azoto*-B-126*	White	Circular	Entire	Convex	_
5.	PSB-S-162**	Yellow	Circular	Undulate	Raised	_
6.	PSB-S-71**	Yellow	Circular	Undulate	Raised	_
7.	PSB-S-64**	White	Irregular	Undulate	Raised	+
8.	PSB-172**	White	Irregular	Undulate	Raised	+
9.	PSB-S-170**	Yellow	Circular	Undulate	Raised	_
10.	PSB-S-87**	White	Irregular	Undulate	Raised	+
11.	PSB-H-5**	White	Irregular	Undulate	Raised	+
12.	PSB-H-27**	Yellow	Circular	Undulate	Raised	_
13.	PSB-S-88**	Yellow	Circular	Undulate	Raised	_
14.	PSB-S-165**	White	Irregular	Undulate	Raised	+

* Grown on Jensen's Agar medium (+) = Positive

** Grown on Pikovskaya's Agar medium (-) = Negative

Table.2 Biochemical tests of isolates

S.No.	Isolate No.	Biochemical characterization							
		Catalase test	TSI Test	Urease test	Citrate test	Indole production test	MR-VP Test		Gelatin Liquefaction test
							MR Test	VP Test	
1.	Azoto-123	-	-	-	+	-	-	-	-
2.	Azoto-B-44	-	+	-	+	-	-	-	-
3.	Azoto-146	-	+	-	+	-	-	-	-
4.	Azoto-B-126	-	+	-	+	-	-	-	-
5.	PSB-S-88	-	+	-	+	-	-	-	+
6.	PSB-H-27	-	+	-	+	-	-	-	-
7.	PSB-S-170	-	+	-	+	-	-	-	-
8.	PSB-172	-	-	-	-	-	+	-	-
9.	PSB-S-64	-	-	-	-	-	+	-	-
10.	PSB-S-71	-	+	-	+	-	-	-	+
11.	PSB-H-5	-	+	-	+	-	-	-	+
12.	PSB-S-165	-	-	-	-	-	-	-	+
13.	PSB-S-162	-	+	-	+	-	-	-	+
14.	PSB-S-87	-	-	-	-	-	-	-	-
15.	Control	-	-	-	-	-	-	-	-

MR=Methyl red (+)= Positive VP=Voges-Proskauer (-)=Negative TSI=Triple sugar iron

Table.3 N-fixing capacity of *Azotobacter* isolates in N-free Jensen's liquid medium

S.No.	Isolate No.	N-fixed (mg N/gm of sucrose)
1.	Azoto-123	2.400
2.	Azoto-146	2.750
3.	Azoto-B-126	3.250
4.	Azoto-B-44	2.050
5.	Control	0.035
	CD (5%)	0.184

Table.4 Phosphorus solubilizing capacity of different PSB isolates

S.No.	Isolate No.	P – solubilized (µg P/ml)
1.	PSB-S-162	375.88
2.	PSB-172	278.43
3.	PSB-S-88	361.76
4.	PSB-H-5	894.51
5.	PSB-S-64	263.72
6.	PSB-S-170	576.47
7.	PSB-S-87	316.67
8.	PSB-S-71	768.08
9.	PSB-H-27	803.92
10.	PSB-S-165	471.57
11.	Control	214.29

	CD (5%)	39.55

Table.5 Solubilization of tri-calcium phosphate by different PSB isolates

S.No.	Isolate No.	Growth diameter (mm)	Solubilization diameter (mm)	Solubilization efficiency (%)
1.	PSB-S-165	7	13	85
2.	PSB-S-162	8	13	62
3.	PSB-S-71	7	13	85
4.	PSB-H-27	7	14	100
5.	PSB-H-5	7	13	85
6.	PSB-S-87	6	11	83
7.	PSB-S-88	8	12	50
8.	PSB-S-170	8	13	62
9.	PSB-172	7	10	42
10.	PSB-S-64	7	10	42
	CD(5%)		2.5	2.8

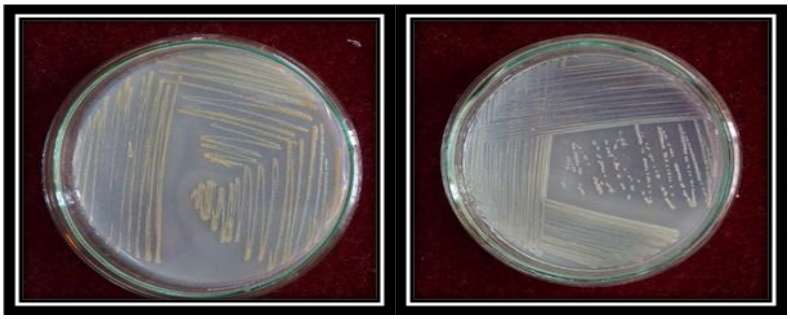
Table.6 Determination of antibiotic susceptibility of different bacterial isolates

S.No.	Isolate No.	Antibiotic							
		Ampicillin (10mcg)		Penicillin (10mcg)		Tetracycline (30mcg)		Streptomycin (10mcg)	
		Sensitivity	Zone dia. (mm)	Sensitivity	Zone dia. (mm)	Sensitivity	Zone dia. (mm)	Sensitivity	Zone dia. (mm)
1.	Azoto-B-126	R	0.0	R	0.0	S	24.0	S	19.0
2.	Azoto-123	R	0.0	R	0.0	S	11.0	R	0.0
3.	Azoto-B-44	R	0.0	R	0.0	R	0.0	S	23.0
4.	Azoto-146	R	0.0	R	0.0	S	17.0	S	18.0
5.	PSB-S-165	R	0.0	R	0.0	R	0.0	R	0.0
6.	PSB-H-5	R	0.0	R	0.0	S	37.0	R	0.0
7.	PSB-S-87	R	0.0	R	0.0	R	0.0	R	0.0
8.	PSB-S-88	R	0.0	R	0.0	R	0.0	R	0.0
9.	PSB-S-170	R	0.0	R	0.0	S	36.0	R	0.0
10.	PSB-172	R	0.0	R	0.0	R	0.0	R	0.0
11.	PSB-H-27	R	0.0	R	0.0	S	38.0	R	0.0
12.	PSB-S-71	R	0.0	R	0.0	S	34.0	R	0.0
13.	PSB-S-162	R	0.0	R	0.0	R	0.0	R	0.0
14.	PSB-S-64	R	0.0	R	0.0	R	0.0	R	0.0
15.	Control	R	0.0	R	0.0	R	0.0	R	0.0

R = Resistant S = Susceptible

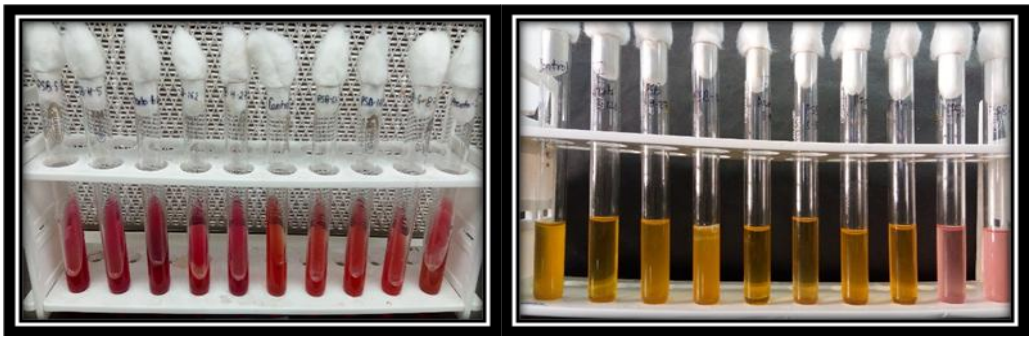
Fig.1 Colony morphology of isolates

Growth of *Azotobacter* on Jensen's media

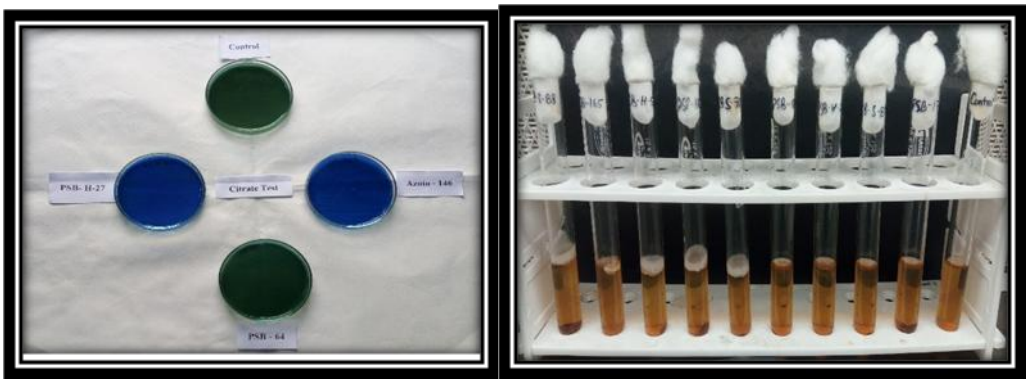


Growth of PSB on Pikovskaya's media

Fig.2 Biochemical characterization of isolates



Triple sugar Iron (TSI) test Methyl red test



Citrate test Gelatin liquefaction test

Fig.3 Growth of solubilization zone by PSB isolates

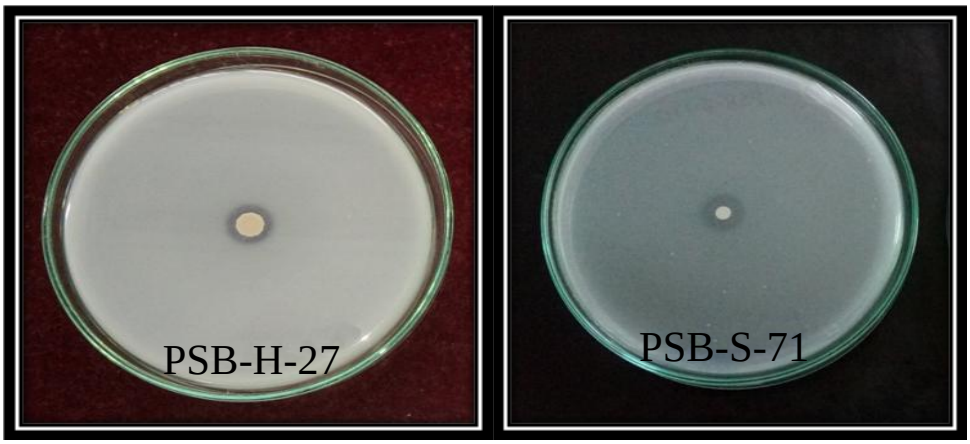
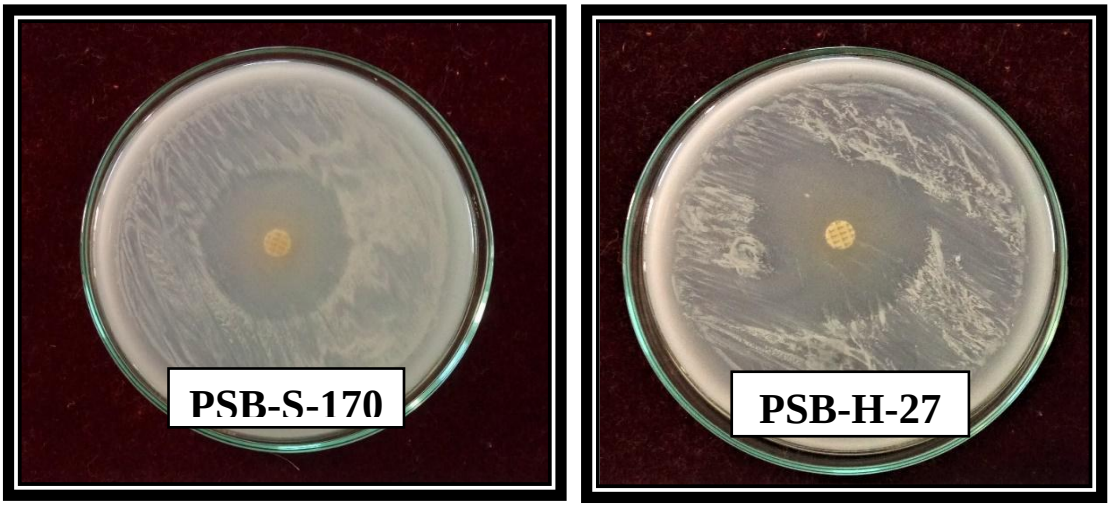
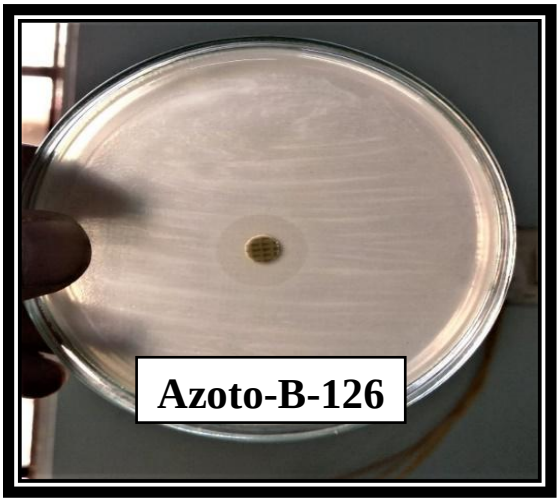


Fig.4 Antibiotic response of isolates





B. cepacia and *B. ferrariae* showed the best activities of solubilization for all source evaluated. The results obtained by *B. ferrariae* confirm that from Valverde *et al.*, (2006), who isolated this organism from rock phosphate mines and considered it a great potential solubilizer.

It was concluded from the present study that native isolates showed variation in their character during different biochemical studies, screening and their antibiotic response. Due to their capacity of nitrogen fixing and phosphorus solubilization, the isolates can be exploited in future as biofertilizers for the improvement of crop productivity.

Acknowledgement

I am thankful to Department of Agricultural Microbiology, Indira Gandhi Krishi Vishwavidyalaya, Raipur, C.G., India for providing all the facilities to conduct my research work.

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How to cite this article:

Hemlata Painkra, Tapas Chowdhury and Narayan Prasad Verma. 2019. Characterization and Screening of Native Isolates of PSB and *Azotobacter* under *In-vitro* Conditions. *Int.J.Curr.Microbiol.App.Sci.* 8(05): 2058-2068. doi: https://doi.org/10.20546/ijcmas.2019.805.239