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IN VITRO CHARACTERIZATION OF PHOSPHATE SOLUBILIZING BACTERIA ISOLATED FROM DIFFERENT AGRICULTURAL FIELDS

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ABSTRACT

The phosphate solubilizing bacteria were isolated from the rhizosphere soils of Cotton, Brinjal, Bhendi, Tomato and Cluster bean. Two PSB isolates from each crop plants totally 10 PSB strains were isolated for further studies. The isolated PSB were identified by their morphological and biochemical properties. The isolated PSB strains were characterized under in-vitro by measuring the P solubilization zone in solid medium, phosphate solubilizing index, determining pH change of the medium and estimating the organic acid production, phosphates activity and available phosphorus. Among ten strains, the BP1 strain was superior in pH reduction, organic acid production, phosphatase activity and more in P release.

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INTRODUCTION

Phosphorus is a plant macronutrient that plays a significant role in plant metabolism, ultimately reflected on crop yields. It is estimated that about 98% of Indian soils contain insufficient amounts of available phosphorus, which is necessary to support maximum plant growth (Tallapragada and Seshachala, 2012). Tropical and subtropical soils are predominantly acidic and often extremely phosphorous deficient (Gaume, 2000) with high phosphorous sorption (fixation) capacities. The low level of phosphorous is due to high reactivity of soluble phosphate with other elements. Biofertilizers are usually prepared as carrier based inoculants containing effective microorganisms. Incorporation of microorganisms in carrier material enables easy-handling, long term storage and high effectiveness of biofertilizers. Among various types of biofertilizers, bacterial inoculant is one major group which includes nitrogen fixing rhizobacteria, plant growth promoting rhizobacteria and phosphate solubilizing bacteria (Packialakshmi and Aliya, 2014; Mondal *et al*., 2017). PSB includes bacteria, fungi, actinomycetes and even algae. Bacteria are predominant amongst them and proved more effective in phosphate solubilization than fungi. Several soil bacteria, particularly those belonging to the genera *Pseudomonas* and *Bacillus* possess the ability to convert the insoluble phosphate into soluble form by secreting organic acids resulting in improved phosphate availability to the plants are called PSB (Fankem *et al.,* 2006). Phosphate solubilizing microbes dissolves the phosphorus content in soil through the formation of organic acids and reduces the pH of the rhizosphere. The solubilization of insoluble phosphate is ability to Secretion of organic acids and phosphatases to solubilize insoluble phosphate to soluble forms are common in this group. Although several phosphate solubilizing bacteria occur in soil, their numbers are not adequate to compete with other bacteria commonly established in the rhizosphere (Khan *et al.,* 2010). The PSBs are able to synthesize phytohormones like indole acetic acid, gibberellic acid (Ramkumar and Kannapiran, 2011) and siderophore (Babna *et al*., 2013). PSBs are also enhances plant growth by increasing the available P and some trace elements such as iron, zinc, *etc*. (Ponmurugan and Gopi, 2006).

MATERIAL AND METHODS

Isolation and Enumeration of PSB: Rhizosphere soil samples were collected from the cultivable agricultural fields plants such as Bhendi, Brinjal, Cotton, Tomato and Cluster

bean. Isolation and enumeration of PSB was carried out following dilution plate technique using hydroxy apatite medium. For the isolation PSB, the rhizoshere soil samples were serially diluted up to 10^6 dilutions and plated on petriplates and incubated at 35±2ºC for seven days. At the end of incubation, PSB colonies were visually identified from the clear zone around the bacterial colony. The colonies were sub cultured, purified and maintained in nutrient agar slants.

Identification of Psb

The isolated bacterial strains were identified using standard biochemical tests as listed in the Bergey's Manual of Determinative Bacteriology (Krieg and Dobereiner, 1984).

Biology of Psb Strains

pH tolerance: The pH tolerance of selected strains of PSB was tested in yeast extract glucose agar medium prepared at pH range of 5.0 to 8.0. The cultures were streaked on the medium and incubated at 35±2°C. After 3 days the growth of PSB strains were observed.

Temperature tolerance: The PSB strains were streaked on Luria Bertaini medium and kept at different temperature (5, 20, 28, 35 and 50ºC) and recorded the nature of growth.

Utilization of carbon, nitrogen, amino acid and vitamin sources: The utilization of different carbon, nitrogen, amino acid and vitamin sources by PSB isolates were estimated in LB broth. Filter sterilized carbon, nitrogen, amino acid and vitamin sources were inoculated aseptically into the sterile medium at 1 per cent level. The PSB cultures were inoculated at the rate of 1.0 ml and incubated at room temperature. The growth was observed by the turbidity of the broth read at 560 nm.

Screening of PSB strains

Phosphate solubilization in the solid medium

The isolated PSB strains were inoculated in solid hydroxy apatite medium and incubated for 7 days. After incubation period the diameter of the halo zone produced around the colonies was measured.

Phosphate Solubilization Index (PSI)

PSB isolates were selected from the colonies based on their ability to form a clear halo on Pikovskaya's agar medium. The isolates were aseptically spot inoculated onto the center of an agar plate. All plates were incubated at 28°±2°C for 7 days. A clear zone around a growing colony indicated phosphate solubilization and was measured as the phosphate solubilization index (PSI). The phosphate solubilizing index was evaluated using the formula derived by Edi-Premono *et al*. (1996).

pH reduction

The selected PSB strains were grown in LB broth and inoculated 1 ml of culture to Pikovskaya's broth. After incubation period the pH was measured.

Estimation of organic acids

The organic acid produced by PSB strain was estimated in terms of total titrable acidity of the culture filtrate (Sperber 1958). PSB strains were inoculated in the Pikovaskaya's broth and allowed to grow for 7 days. After incubation period the culture was centrifuged and removed the cell biomass. Two milliliter of culture filtrate was taken in a flask and added few drops of phenolphthalein and titrated against 0.01 N of sodium hydroxide. The volume of alkali consumed by culture filtrate was the total titrable acidity of the culture filtrate. The total titrable acidity was expressed by ml of 0.01 N NaOH consumed.

Estimation of phosphatase activity

Phosphatase activity was estimated by method described by Eivazi and Tabatabai (1977). From this, centrifuged the culture broth at 10,000 rpm for 10 minutes and the pellet was suspended in 5 ml of sterile distilled water. 1 ml of the sample was taken in a 50 ml conical flask and added 0.25 ml of toluene, 4 ml of modified universal buffer, 1 ml of p-nitrophenyl phosphate solution and incubated at room temperature for 1 h. After incubation, add 1 ml of 0.5 M of calcium chloride and 4 ml of 0.5 M sodium hydroxide and filtered the content through a filter paper. The absorbance was measured at 660 nm. The phosphatase activity was expressed as μ moles of PNP released ml^{-1} of filtrate h^{-1}.

Estimation of available phosphorus

The available phosphorus in the culture filtrate was estimated following the method of Olsen *et al.* (1954). The broth culture was centrifuged at 1000 rpm for 10 minutes and the clear supernatant was used for the estimation of available phosphorus. One milliliter of culture filtrate was pipetted out into a 25 ml standard flask and added 10 ml of distilled water and shake well. Then 5 ml of freshly prepared ascorbic acid and ammonium molybdate solution were added and made up to 25 ml. Absorbance was measured at 882 nm.

RESULTS

Isolation and population dynamics of Psb

The population level of PSB was higher in the rhizosphere soils of cotton and least in brinjal and cluster bean. Based on the solubilization zone production in the hydroxy apatite solid medium, two PSB isolates from each crop plant totally 10 PSB strains were isolated. The isolated 10 PSB strains were subcultured regularly and used for further studies (Table 1).

Identification of psb strains

Based on the morphological and biochemical tests, the PSB strains were identified up to species level. The PSB isolates, BDP1, BP1, CP2, TP1, CBP2 were identified as *Bacillus megaterium* while BDP2, BP2, CP1, TP2, CBP1 as *Pseudomonas putida* (Table 2). *Bacillus megaterium* were positive to catalase, acid from glucose, casein hydrolysis, gelatin hydrolysis, starch hydrolysis, citrate utilization, nitrate reduction and growth at pH 6-8 on nutrient broth and negative to anaerobic growth, gas from glucose, VP test and indole production. *Pseudomonas putida* were positive to oxidase, catalase, arginine dihydrolase, acid from glucose and growth on citrate agar and negative to growth at 41ºC, gelatin hydrolysis, starch hydrolysis and denitrification.

Biology of Psb Strains

The PSB strains preferred temperature ranging from 20°C to 35^0C and above and below which the growth was retarded.

PSB strains could grow well at temperature 28^0C to 35^0C (Table 3). The optimum pH for the growth of PSB was ranged from 6.0 to7.5 (Table 4). The PSB strains utilized different types of chemical compounds as carbon source.

Table 1. Population level of PSB in the rhizosphere soils of different crop plants

S.No.	Crop Plants	Population level ($\times 10^5$/g soil dry wt.)	PSB isolates and their Code Number
1.	Bhendi	2.0	BDP1
			BDP2
2.	Brinjal	1.5	BP1
			BP2
3.	Cotton	7.5	CP1
			CP2
4.	Tomato	4.3	TP1
			TP2
5.	Cluster bean	1.5	CBP1
			CBP2

Table 2. Identified PSB strains

S. No.	PSB Strains	Identified PSB
1	BDP1	*Bacillus megaterium*
2	BDP2	*Pseudomonas putida*
3	BP1	*Bacillus megaterium*
4	BP2	*Pseudomonas putida*
5	CP1	*Pseudomonas putida*
6	CP2	*Bacillus megaterium*
7	TP1	*Bacillus megaterium*
8	TP2	*Pseudomonas putida*
9	CBP1	*Pseudomonas putida*
10	CBP2	*Bacillus megaterium*

Table 3. Temperature tolerance of PSB

PSB Strains	Temperature				
	5^oC	20^oC	28^oC	35^oC	50^oC
BDP1	-	++	+++	+++	-
BDP2	-	++	+++	+++	-
BP1	-	++	+++	+++	-
BP2	-	++	+++	+++	-
CP1	-	++	+++	+++	-
CP2	-	++	+++	+++	-
TP1	-	++	+++	+++	-
TP2	-	++	+++	+++	-
CBP1	-	++	+++	+++	-
CBP2	-	++	+++	+++	-

(-) no growth (+) poor growth (++) medium growth (+++) good growth

The utilization of carbons sources varied from strain to strain. Most of the PSB strains were preferred well glucose and fructose as carbon source and maltose, sucrose and lactose were moderately utilized. Further, starch were poorly utilized (Table 5). It was observed that PSB strains moderately utilized the nitrogenous compounds such as ammonium nitrate, ammonium chloride, potassium nitrate and urea. Further, ammonium sulphate was poorly utilized by PSB strains (Table 6). All the aminoacids were found to be supported the growth of PSB strains. Aminoacids like alanine and therionine were preferred more compared to other aminoacids (Table 7). Among different types of vitamins tested, ascorbic acid was utilized more by PSB strains. Biotin and myoinositol were moderately utilized and nicotinic acid and thiamine poorly utilized by all PSB strains (Table 8).

Table 4. pH tolerance of PSB

PSB Strains	pH				
	5.0	6.0	7.0	7.5	8.0
BDP1	+	++	+++	+++	+
BDP2	+	++	+++	+++	+
BP1	+	++	+++	+++	+
BP2	+	++	+++	+++	+
CP1	+	++	+++	+++	+
CP2	+	++	+++	+++	+
TP1	+	++	+++	+++	+
TP2	+	++	+++	+++	+
CBP1	+	++	+++	+++	+
CBP2	+	++	+++	+++	+

Table 5. Utilization of carbon sources by PSB

PSB Strains	Glucose	Fructose	Maltose	Sucrose	Lactose	Starch
BDP1	+++	+++	++	++	++	+
	(1.298)	(1.412)	(0.652)	(0.512)	(0.618)	(0.359)
BDP2	+++	+++	++	++	++	+
	(1.290)	(1.328)	(0.587)	(0.651)	(0.572)	(0.217)
BP1	+++	+++	++	++	++	+
	(1.319)	(1.254)	(0.886)	(0.619)	(0.932)	(0.427)
BP2	+++	+++	++	++	++	+
	(1.250)	(1.356)	(0.964)	(0.910)	(0.748)	(0.419)
CP1	+++	+++	++	++	++	+
	(1.215)	(1.453)	(0.848)	(0.577)	(0.608)	(0.492)
CP2	+++	+++	++	++	++	+
	(1.318)	(1.369)	(0.958)	(0.706)	(0.619)	(0.304)
TP1	+++	+++	++	++	++	+
	(1.179)	(1.354)	(0.761)	(0.621)	(0.795)	(0.402)
TP2	+++	+++	++	++	++	+
	(1.165)	(1.208)	(0.608)	(0.859)	(0.917)	(0.329)
CBP1	+++	+++	++	++	++	+
	(1.330)	(1.367)	(0.803)	(0.994)	(0.832)	(0.426)
CBP2	+++	+++	++	++	++	+
	(1.250)	(1.292)	(0.856)	(0.529)	(0.778)	(0.261)

Values in parentheses indicate OD value
(+)-0.0-0.5 (Poor Growth); (++) - 0.51-1.0 (Moderate Growth); (+++) -1.1-1.5 (Best Growth)

Table 6. Utilization of nitrogen compounds by PSB

PSB Strains	Ammonium nitrate	Ammonium sulphate	Ammonium chloride	Potassium nitrate	Urea
BDP1	++ (0.926)	+ (0.506)	++ (0.682)	++ (0.781)	++ (0.597)
BDP2	++ (0.766)	+ (0.425)	++ (0.556)	++ (1.021)	++ (0.582)
BP1	++ (1.064)	+ (0.430)	++ (0.578)	++ (0.743)	++ (0.712)
BP2	++ (0.965)	+ (0.364)	++ (0.732)	++ (0.571)	++ (1.008)
CP1	++ (1.021)	+ (0.457)	++ (0.794)	++ (1.008)	++ (1.078)
CP2	++ (0.824)	+ (0.443)	++ (0.633)	++ (0.678)	++ (1.048)
TP1	++ (0.900)	+ (0.329)	++ (0.702)	++ (1.046)	++ (0.876)
TP2	++ (0.524)	+ (0.483)	++ (0.554)	++ (0.875)	++ (0.617)
CBP1	++ (0.794)	+ (0.346)	++ (0.681)	++ (0.619)	++ (0.954)
CBP2	++ (0.632)	+ (0.504)	++ (0.521)	++ (0.632)	++ (0.552)

Table 7. Utilization of aminoacids by PSB

PSB Strains	Leucine	Isoleucine	Alanine	Therionine	Cytosine
BDP1	++ (0.578)	++ (0.624)	+++ (1.214)	+++ (1.116)	++ (0.749)
BDP2	++ (0.656)	++ (0.820)	+++ (1.384)	+++ (1.489)	++ (0.567)
BP1	+++ (1.143)	++ (0.765)	+++ (1.302)	+++ (1.123)	++ (0.522)
BP2	++ (1.038)	++ (0.515)	+++ (1.143)	+++ (1.229)	++ (0.758)
CP1	++ (1.027)	++ (0.796)	+++ (1.152)	+++ (1.148)	++ (0.566)
CP2	+++ (1.240)	++ (0.892)	+++ (1.264)	+++ (1.159)	++ (0.848)
TP1	++ (0.706)	++ (0.534)	+++ (1.156)	+++ (1.318)	++ (0.592)
TP2	++ (1.012)	++ (0.627)	+++ (1.237)	+++ (1.122)	++ (0.861)
CBP1	++ (0.534)	+++ (1.178)	+++ (1.148)	+++ (1.278)	++ (0.583)
CBP2	++ (1.023)	++ (1.069)	+++ (1.287)	+++ (1.286)	++ (0.956)

Table 8. Utilization of vitamins by PSB

PSB Strains	Nicotinic acid	Thiamine	Ascorbic acid	Myoinositol	Biotin
BDP1	+ (0.426)	+ (0.482)	+++ (1.282)	++ (0.763)	++ (0.627)
BDP2	+ (0.393)	+ (0.465)	+++ (1.102)	++ (0.645)	++ (0.708)
BP1	+ (0.364)	+ (0.373)	+++ (1.376)	++ (0.573)	++ (0.826)
BP2	+ (0.384)	+ (0.495)	+++ (1.298)	++ (0.826)	++ (1.048)
CP1	+ (0.342)	+ (0.484)	+++ (1.387)	++ (0.600)	++ (0.964)
CP2	+ (0.283)	+ (0.316)	+++ (1.145)	++ (1.023)	++ (1.086)
TP1	+ (0.463)	+ (0.486)	+++ (1.368)	++ (0.634)	++ (0.527)
TP2	+ (0.348)	+ (0.395)	+++ (1.284)	++ (0.725)	++ (0.744)
CBP1	+ (0.235)	+ (0.493)	+++ (1.302)	++ (0.862)	++ (0.648)
CBP2	+ (0.103)	+ (0.327)	+++ (1.282)	++ (1.035)	++ (1.056)

Screening of psb Strains

The isolated PSB strains were screened under *in vitro* to select elite strain. Phosphate solubilization zone produced by PSB was estimated by measuring the solubilization zone in the hydroxyl apatite solid medium.

matter, salinity level and some enzyme activities in water column. Seshadri *et al*. (2002) carried out an investigation on microbial dynamics in the water column reported that there was a significant difference on the population level of PSB in different places.

Table 9. Characterization of PSB

PSB Strains	Solubilizing zone (mm)	Phosphate Solubilization Index (PSI)	pH Reduction	Organic acid (0.1 N NaOH Consumed)	Phosphatase activity (µ mole/ml/hr)	Available P (ppm)
BDP1	4	2.60	5.0	5.1	15.3	32.8
	±0.34	±0.03	±0.49	±0.10	±0.68	±0.50
BDP2	3	2.42	4.2	4.3	18.0	30.5
	±0.18	±0.02	±0.34	±0.04	±0.71	±0.35
BP1	6	3.30	3.7	13.0	23.2	40.0
	±0.45	±0.05	±0.33	±0.30	±0.92	±0.68
BP2	3	2.76	3.9	9.8	22.8	33.5
	±0.22	±0.03	±0.14	±0.25	±0.77	±0.45
CP1	4	2.73	4.5	7.3	18.3	35.2
	±0.24	±0.01	±0.41	±0.21	±0.55	±0.55
CP2	4	2.48	4.8	7.0	13.1	32.6
	±0.22	±0.01	±0.15	±0.20	±0.15	±0.41
TP1	2	2.05	4.6	5.2	16.4	28.7
	±0.11	±0.02	±0.21	±0.05	±0.75	±0.41
TP2	5	3.22	4.2	6.0	13.6	25.3
	±0.34	±0.03	±0.06	±0.15	±0.25	±0.30
CBP1	2	2.25	4.3	4.0	14.3	21.4
	±0.12	±0.01	±0.07	±0.27	±0.30	±0.20
CBP2	2	2.23	5.2	4.7	15.7	20.9
	±0.10	±0.01	±1.07	±0.04	±0.73	±0.15

The solubilization zone was maximum with BP1 strain followed by TP2. To revealed that the strain, BP1 showed maximum phosphate solubilizing index and minimum was recorded in TP1. The maximum pH reduction was noticed with BP1strain followed by BP2 with tricalcium phosphate (TCP) as phosphate source. Among 10 PSB strains, the strains PB1 were good organic acid production (13.0 0.1N NaOH consumed) and least in CBP1 (4.0 0.1N NaOH consumed). Estimation of phosphatase activity indicated that the activity by PSB was higher with the strain BP1 (23.2 µmole/ml/hr) and least with CP2 (13.1 µ mole/ml/hr). The strain BP1 was released more phosphorus in the medium followed by CP1 with TCP as phosphate source (Table 9). Based on the preliminary screening, the PSB strain, BP1was selected as best one.

DISCUSSION

Isolation and Population Dynamics of psb: Based on the solubilization zone around the bacterial colonies, totally 10 PSB strains were isolated from the rhizosphere soil of different crop plants such as Bhendi, Brinjal, Cotton, Tomato and Cluster bean. Karpagam and Nagalakshmi (2014) repoted that PSB strains were isolated using the Pikovskaya's medium based on the formation of halo zone around the microorganisms. The clear or halo zone was formed due to the solubilization of insoluble phosphates by bacteria isolated from different sources (Reena *et al*., 2013).

The population level of PSB was varied in different rhizosphere soil (Kucey, 1983). Soil samples were collected from sugarcane growing belt of north Bihar indicated that the population level of phosphate solubilizing bacterial range from 27-112 x 10^{-31}. This large variation in the distribution of PSB in different soils may be due to the differences in organic carbon content of the soil. The population of PSB might be attributed to many factors such as nutrients, pH, organic

The population of PSB in the rhizosphere of broad bean was more population compare to wheat plant (Gianfreda, 2015). Mahmoud *et al*. (1973) made a comparative study of the population of PSM in the rhizosphere of broad bean and wheat. Broad bean had more population of phosphate solubilizers than the wheat plants.

Identification of psb strains

Gaur (1990) studied the bacterial cultures morphological, physiological and biochemical characteristics using the manual of microbiological methods and identified the organisms *Bacillus* sp. and *Pseudomonas* sp. using Bergey's Manual of Determinative Bacteriology. Sankaralingam *et al*. (2014) isolated eight phosphate solubilizing bacteria from the rhizosphere soil of weed plants. Among the eight phosphate solubilizing organisms, the efficient phosphate solubilizer was identified as *Enterobacter canaerogenus* based the morphological and biochemical character. Richardson (2001) found that the predominant soil bacteria involved in phosphate solubilization include *Bacillus* and *Pseudomonas*. Kumar and Tripathi (2011) collected soil sample for their study from rhizosphere of four different types of plants of Aloe vera, Mango, Graveyard, also included park soil, from which they isolated *Pseudomonas fluorescens* and *Bacillus megaterium*. Predominant PSB isolates identified were *P. aeruginosa* and *Bacillus subtilis*. All PSB isolates utilized sugars oxidatively, forming acid only. Indole, MR, VP, were negative except that A2 was citrate positive and A1 as citrate negative. Both were catalase positive and urease negative. On the basis of above observation it was found that the strains were identical morphological, biochemical and cultural characteristics like that *Bacillus subtilis* and *Pseudomonas aeruginosa* (Bhoosreddy, 2014).

Biology of PSB

Tolerance to pH and temperature, utilization of carbon, nitrogen, vitamin and aminoacid sources varied within the

selected strains of PSB. These variations are mainly due to the nature of strains and differences in the species of respective genus. The optimization of incubation temperature plays a major role in the growth of the microorganism as the microorganisms are metabolically active at optimum temperature. The optimization temperature of the isolates *Bacillus megaterium* and *Pseudomonas aeruginosa* was carried out where in the isolates showed maximum growth at the incubation temperature of 33ºC and 37ºC respectively (Prasad, 2014). The optimization of the pH was carried out to check the maximum growth of the phosphate solubilizing bacteria at different pH value, where in both *Bacillus megaterium* and *Pseudomonas aeruginosa* proved to grow luxuriantly at a Ph value of 7.2 (Sane and Mehta, 2015). The form of available carbon sources greatly affected the growth as well as the phosphate solubilization under *in vitro* and *in situ* in soil and was more active in presence of hexoses and pentoses or dissacharides. The nitrogen sources supported the growth and P solubilization of *Pseudomonas fluorescence* (Dave and Patel, 2003). Gyaneshwar *et al*. (1998) found that *Enterobacter asburiae* solubilized phosphate when grown in presence of ammonia and nitrate as nitrogen source. Likewise, all the amino acids and vitamins supported the growth of PSB strains but the preference differed from strains to strains.

Screening of Psb Strains

P Solubilization zone and pH reduction: A clear or halo zone was formed by PSB due to the solubilization of insoluble phosphates by acidification of association of either proton extrusion or organic acid secretion. Phosphate solubilization potential has been attributed to the strains ability to reduce pH of the surroundings, either by releasing organic acids or protons (Hariprasad and Niranjana, 2009). The lowering in pH of the medium suggests the secretion of organic acids by the P-solubilizing microorganisms (Maliha *et al*., 2004) *via* direct oxidation pathway that occurs on the outer face of the cytoplasmic membrane (Zaidi *et al*., 2009). The maximum decline in pH was recorded with *Bacillus megaterium* from 6.0 to 4.2 and *B. cerus* from 6.6 to 5.6. A fall in pH accompanied phosphate solubilization due to the production of organic acids, but no correlation could be established between acidic pH and quantity of P_2O_5 liberated. The PSB isolates, PKS 4, PBS 4, PKS 3 and PKU 5 significantly decreased the pH of media up to 3.3, 3.6, 3.8, 3.6 and 3.4 respectively after 7^{th} day of growth (Fatima *et al.,* 2015).

Phosphate Solubilizing Index: The isolated PSB produced very large zone size (30-45mm) on chitinase media screening of the most efficient PSB *in vitro* was based on the ability of the isolate to release phosphorus into the culture medium and its relationship with the phosphate solubilizing index (PSI) based on colony diameter and halozone for each isolate. PSI were positive but not significant correlation was observed between qualitative and quantitative P- solubilization. This finding was in support to the moderate positive correlation between the TCP solubilizing efficiency on solid medium and amount of phosphate solubilized in liquid medium by *Pseudomonads* and least correlation was found with P-solubilization and halo zone diameter (Srivastav *et al*., 2004). The PSB isolates were found to be potent phosphate solubilizers showing clear zone around their colonies. Out of 37 isolates, 6 isolates showed highest PSI ranged from 1.13 - 2.23. Among these 6 potent isolates, 3 strains showed maximum PSI (Karpagam and Nagalakshmi, 2014). Sanjotha and Manawadi (2016) isolated, 35 PSB strains from 15 soil samples and the samples were screened for phosphate solubilization ability, of these 5 microbial isolates showed highest PSI ranged from 2.0 - 2.63. The microbial colonies showing clear halo zones around the microbial growth were considered as phosphate solubilization. Generally bacterial isolates with clear halozone around the colony were selected. Solubilization index based on colony diameter and halozone formation for each isolates were determined on PKA agar plates. Generally it was observed that, as colony diameter increases zone of clearance around colony also increases by solubilizing tricalcium phosphate in the medium (Shankarrao, 2012).

Organic acid production: The production of organic acids by PSB seems to the main reason for solubilization of P and some metabolites like CO_2, H_2S and alkalinity or HNO_3 produced by organotrophs or autotrophs were also implicated to a little extent in P solubilization (Illmer and Schinner, 1995). P-solubilizing activity were related to the microbial production of organic acids, which chelate the cation bound to phosphate, thereby converting it to a soluble form. Organic acids such as gluconic acid, oxalic acid and citric acid, secreted by PSB can directly solubilize mineral phosphate of anion exchange or indirectly chelate both Fe and Al ions associated with phosphate. This leads to increased P availability, which ultimately increased the P leval (Gnana Sankar *et al*., 2017). Phosphate solubilizing bacteria isolated from the crops grown in vertisols for the production of organic acids such as fumaric, citric, gluconic, maleic, tartaric, succinic acids and other unidentified acids (Vikram *et al*., 2007). The type of organic acid produced and their amounts differ with different organisms. Among them, gluconic acid and 2- ketogluconic acid seems to be the most frequent agent of mineral phosphate solubilization (Song *et al*., 2008).

Phosphatase activity: Roca *et al*. (2013) found that the logic behind higher alkaline phosphatase enzyme activity was presence of insoluble phosphate for which PSB can produce enzymes and organic acids to solubilize this unavailable phosphorus. The PSB were grown on Pikovskaya's broth containing 0.5 % TCP and their phosphatase activities were measured. The main mechanism for the solubilization of insoluble organic and inorganic phosphate was due to production of an enzyme acid phosphatase, which catalyzes hydrolysis of phosphate to liberate inorganic phosphorus (Pi). Thus, the isolates were evaluated for their acid phosphatase producing ability by measuring Pi ability. Among all the positive isolates, six bacteria exhibited significantly higher amount of acid phosphatase enzyme activity. Moreover, these isolates also showed relatively higher solubilization index. Thus, the solubilization index and the acid phosphatase enzyme activity were directly proportional to each other indicating that high enzymatic activity results in the formation of large halo zone (Sheng *et al.,* 2002). There was a positive correlation between phosphate solubilizing capacity and phosphatase activity. This might be due to availability of higher amount of P in the medium and the ability of the strains (Barik and Purushothaman, 1998).

Available Phosphorus: Phosphobacterial strains showed different abilities to release soluble phosphate from rock phosphate. The most efficient strain released 25.87 µg/ml soluble P in the growth medium (Hamdali *et al*., 2011). Generally, available phosphorus in soil can be increased by

low molecular mass organic acids produced from PSB. The P solubilization potential of selected strains of PSB was tested *in vitro* by estimating available phosphorus in the culture medium. Alternative possibilities other than organic acids for mineral phosphate solubilization have been proposed based on the lack of a linear correlation between pH and the amount of solubilized P. Phosphate solubilizing bacteria produced more available P by the production of organic acids which act like chelates and solubilized insoluble phosphorus. Phosphorus solubilization ability of PSB has direct correlation with pH of the medium. The decrease in phosphate solubilizing ability after a certain period of incubation in both Pikovskaya's medium may be due to production of certain toxic metabolites during late log or decline phase or due to autolysis of cells as suggested by Trivedi and Tongmin (2008).

Conclusion

PSB strains were isolated from different agricultural fields and characterized under *in vitro* studies. The isolated strains were able to utilize various chemical compounds as sources. The strains differed in their utilization capacity. Based on the biochemical tests, the PSB strains were identified up to species level. The selection of elite PSB strain depends on the *in vitro* studies. Based on the *in vitro* studies, the PSB strain BP1 was superior in P solubilization compared to other strains.

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