World Journal of Pharmaceutical and Life Sciences
WJPLS
www.wjpls.org
SJIF Impact Factor: 5.088

CHARACTERIZATION OF PHOSPHATE SOLUBILIZING BACTERIA (PSB) ISOLATED FROM RHIZOSPHERE SOILS OF SELECTED CROP PLANTS

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Article Received on 10/09/2018 **Article Revised on 01/10/2018** **Article Accepted on 22/10/2018**

ABSTRACT

The rhizosphere soils were collected from economically important crop plants such as sunflower, cotton, chilly, tomato, black gram, sorghum, brinjal, green gram, ladys' finger and red gram. Isolation and enumeration of PSB was carried out following dilution plate technique using hydroxy apatite medium. The isolated PSB strains were screened *in vitro* by measuring the P solubilization zone in solid medium, determining pH change of the medium and estimating the phosphatase activity, organic acids production and available phosphorus. The population level of PSB was higher in the rhizosphere soil collected from green gram followed by sunflower. Based on the biochemical and morphological tests, PSB were identified at genus level. The selected strains differed in the solubilization zone formation in the solid medium, pH reduction in the culture medium, production of phosphatase enzyme, organic acids and available phosphorus.

KEY WORDS: Crop plants, rhizosphere, PSB, identification**,** characterization.

INTRODUCTION

Phosphorus is a vital 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.[1] Only about 25% of the phosphorus applied to the soils is available for the crops in the year of its application and remaining part is converted into insoluble unavailable forms. The soil is rich in phosphorus as it contains about 0.05 per cent phosphorus but only one tenth of this is available to plants due to its poor solubility and chemical fixation in soil by aluminium, iron, magnesium *etc*.[2]

Indian soils are rich in P but more than 2.3 of the native phosphates are in a chemical form that cannot be absorbed by plants. Furthermore, applied P fertilizers are rendered unavailable due to its chemical fixation in the soil.[3] Phosphorus is applied to soil in the form of phosphatic fertilizers. However, a large portion of soluble inorganic phosphate applied to the soil as chemical fertilizer is immobilized rapidly and becomes unavailable to plants.[4] Tropical and subtropical soils are predominantly acidic and often extremely phosphorous deficient[5] with high phosphorous sorption (fixation) capacities. The low level of phosphorous is due to high reactivity of soluble phosphate with other elements. Plants acquire P from soil solution as phosphate anions. However, phosphate anions are extremely reactive and may be immobilized through precipitation with cations such as Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+}, depending on the particular properties of a soil. In these forms, P is highly insoluble and unavailable to plants. As the results, the amount available to plants is usually a small proportion of this total. Several scientists have reported the ability of different bacterial species to solubilize insoluble inorganic phosphate compounds such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite and rock phosphate. Several reports have examined the ability of different bacterial species to solubilize insoluble inorganic phosphate compounds.

Phosphate Solubilizing Bacteria (PSB)

Phosphate solubilizing bacteria play an important role in converting low grade insoluble inorganic phosphate sources like rock phosphate, bone meal, basic slag and the chemically fixed soil phosphorus into available form. Therefore, the use of phosphate solubilizing microbes in agricultural practice would not only offset the high cost of manufacturing phosphatic fertilizers but would also mobilize insoluble phosphorus in the fertilizers and soils to which they are applied. The mechanism of solubilization of insoluble phosphate is ability to secrete organic acids and phosphatase enzyme.[6]

PSB have attracted attention in semi arid regions and endowed to enhance the crop yields. They have established their role under nutrient inequity conditions. 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.[7] The PSBs are able to synthesize phytohormones like indole acetic acid, gibberellic acid and siderophores.[8,9] PSB are also enhances plant growth by increasing the efficiency of biological nitrogen fixation of other trace elements such as iron, zinc, *etc*.[10] Phytohormones play an important role as growth regulator such as auxins and cytokinin are responsible for division, extension and differentiation of plant cells and tissues.[11]

MATERIAL AND METHODS

Collection of samples

Soil and root samples were collected from economically important crop plants such as sunflower, cotton, chilly, tomato, black gram, sorghum, brinjal, green gram, ladys' finger and red gram. The soil samples were air dried under shade and used for the isolation and enumeration of PSB.

Isolation and enumeration of PSB

Isolation and enumeration of PSB was carried out following dilution plate technique using hydroxy apatite medium. For the isolation of PSB, the soil samples were serially diluted up to 10^6 dilution 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.

Characterization of PSB strains

Phosphate solubilization in the solid medium

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

Change in pH of the medium

Selected PSB strains were grown in LB broth and inoculated 1 ml to Pikovskaya's broth. After incubation period the pH was measured at different period of growth.

Estimation of organic acids

The organic acid produced by PSB strain was estimated in terms of total titrable acidity of the culture filtrate.[12] The total titrable acidity was expressed by ml of 0.01 N NaOH consumed.

Estimation of phosphatase activity

The PSB isolates were grown in Pikovaskaya's broth where TCP was replaced with organic source (p-glycerophosphate).[13] The phosphatase activity was expressed µ moles of PNP released/ml of filtrate/hour.

Estimation of available Phosphorus

The available phosphorus in the culture filtrate was estimated following the method of Olsen. [14]

Identification of bacterial strains

The selected bacterial strains were identified using standard biochemical tests as listed in the Bergey's Manual of Determinative Bacteriology.[15]

RESULTS

Isolation and population dynamics of PSB

Based on the solubilization zone production in the solid medium, two isolates from each crop plants were selected and totally 20 PSB were isolated. These 20 PSB strains were used for further studies. The isolated PSB strains were maintained on the nutrient agar slants for further use. The population level of PSB was higher in the rhizosphere soil collected from green gram followed by sunflower. The population level of PSB was least in rhizosphere soil collected from the brinjal (Table 1).

Identification of PSB strains

Based on the biochemical and morphological tests, PSB were identified at genus level. Among 20 PSB strains, 9 strains (SFP1, SFP2, CP1, CP2, CHP1, CHP2, TP2, BP1 and RGP1) were identified as *Bacillus spp.* and remaining 11 strains as (SHP1, SHP2, TP1, BGP1, BGP2, BP2, GGP1, GGP2, LP1, LP2, RGP2) *Pseudomonas spp* (Table 2).

Characterization of PSB

The isolated PSB strains were characterized *in vitro* by measuring the P solubilization zone in solid medium, determining pH change of the medium and estimating the phosphatase activity, organic acids production and available phosphorus. The nature of phosphate sources greatly affected P solubilization capacity of PSB strains. Further, the phosphate solubilization capacity was varied with strains to strains (Table 3 and 4).

pH reduction in the culture medium

All the PSB strains brought down the pH of the culture medium with tricalcium phosphate (TCP) and rock phosphate (RP) as phosphate source. Among 20 PSB strains, maximum reduction was noticed with SHP2 and RGP2 strains in the presence of TCP and strains BP2 and RGP2 the presence of RP.

Solubilization zone production by PSB

The Phosphate solubilization zone was estimated by measuring the solubilized zone produced by PSB stains in the solid medium. The P solubilization zone was maximum with the strains CHP1, BP2 and GGP1 in the presence of TCP and strains TP2, BP2 and RGP2 superior in the zone formation in the presence of RP.

Organic acid production

In the culture medium, the addition of TCP as P source, the organic acid production was higher with the strain SHP1 followed by CHP1. In the case of RP, the strains RGP2 and BP2 were superior in organic acid production.

Phosphatase activity

The results revealed that estimation of phosphatase activity indicated that the activity was higher with TCP by CHP1 followed by SP2 strain. Further, the activity was superior with RP by BP2 and RGP2 strains.

Available Phosphorus

The P solubilization potential of selected strains of PSB was tested *in vitro* by estimating available phosphorus in the culture medium. The results indicated that a wide variation in the phosphate solubilization capacity with different strains in presence of both TCP and RP. Among 20 PSB strains, the strains CHP1 was released more phosphorus in the culture medium followed by SHP2 with TCP and BP2 followed by RGP2.

Table 1: Population dynamics of phosphate solubilizing bacteria.

S. No.	Sample	Code Number	Population level
			PSB (x 10^5 g. soil dry wt.)
1.	Sunflower	SFP1 SFP2	4.21
2.	Cotton	CP1 CP2	2.53
3.	Chilly	CHP1 CHP2	1.09
4.	Tomato	TP1 TP2	2.38
5.	Black gram	BGP1 BGP2	1.03
6.	Sorghum	SP1 SP2	0.53
7.	Brinjal	BHP1 BHP2	0.19
8.	Green Gram	GGP1 GGP1	4.59
9.	Okra	LP1 LP2	0.44
10.	Red Gram	RGP1 RGP2	2.75

Table 2: Identification of Bacterial isolates.

S. No.	PSB Strains	Identified strains
1	SFP1	*Bacillus* sp.
2	SFP2	*Bacillus* sp.
3	CP1	*Bacillus* sp.
4	CP2	*Bacillus* sp.
5	CHP1	*Bacillus* sp.
6	CHP2	*Bacillus* sp.
7	TP1	*Pseudomonas* sp.
8	TP2	*Bacillus sp.*
9	BGP1	*Pseudomonas* sp.
10	BGP2	*Pseudomonas* sp.
11	SHP1	*Pseudomonas* sp.
12	SHP2	*Pseudomonas* sp.
13	BP1	*Bacillus* sp.
14	BP2	*Pseudomonas* sp.
15	GGP1	*Pseudomonas* sp.
16	GGP2	*Pseudomonas* sp.
17	LP1	*Pseudomonas* sp.
18	LP2	*Pseudomonas* sp.
19	RGP1	*Bacillus* sp.
20	RGP2	*Pseudomonas* sp.

Table 3: Characterization of PSB with Tri-Calcium Phosphate (TCP) as P source.

PSB Strains	pH Reduction	Solubilization zone formation (mm)	Organic production (0.1 N NaOH consumed)	Phosphatase Activity (µmoles/ml/hr)	Available P (ppm)
SFP1	4.26	3	5.8	12.30	26.25
SFP2	4.14	5	10.0	25.60	42.00
CP1	4.42	3	6.4	13.80	27.25
CP2	4.38	4	5.9	13.40	33.25
CHP1	4.09	6	10.5	25.90	44.30
CHP2	4.47	3	5.0	11.50	33.20
TP1	4.34	4	5.5	13.70	26.75
TP2	4.34	4	5.8	16.80	29.25
BGP1	4.54	2	7.7	19.10	36.75
BGP2	4.36	3	6.0	15.40	28.50
SHP1	4.20	5	8.5	19.80	36.75
SHP2	4.03	5	11.2	24.50	42.75
BHP1	4.65	3	9.4	12.30	32.80
BHP2	4.33	6	8.3	15.60	37.80
GGP1	4.20	6	6.0	16.80	39.30
GGP2	4.26	5	6.4	16.10	37.80
LP1	4.26	4	3.9	15.90	32.50
LP2	4.43	4	4.8	14.10	34.90
RGP1	4.91	3	6.4	11.50	22.30
RGP2	4.04	5	10.8	26.40	40.63

Table 4: Characterization of PSB with Rock Phosphate (RP) as P source.

PSB Strains	pH Reduction	Solubilization zone formation (mm)	Organic production (0.1 N NaOH consumed)	Phosphatase Activity (µmoles/ml/hr)	Available P (ppm)
SFP1	4.7	3	4.0	14.3	22.5
SFP2	4.7	3	4.3	9.8	18.5
CP1	4.8	3	4.5	12.0	28.0
CP2	4.3	4	6.0	12.0	26.5
CHP1	4.3	5	5.7	18.0	30.2
CHP2	4.6	4	4.8	12.5	21.8
TP1	4.5	3	5.3	14.8	18.8
TP2	4.4	6	5.6	13.5	24.5
BGP1	4.4	5	8.0	18.8	29.7
BGP2	4.4	4	7.8	13.5	23.5
SHP1	4.7	3	3.8	9.0	20.3
SHP2	4.5	2	5.4	12.8	28.6
BP1	5.0	2	4.2	11.0	22.3
BP2	4.0	6	8.3	19.5	45.6
GGP1	4.4	4	5.4	19.3	21.5
GGP2	4.3	5	5.5	11.3	25.6
LP1	4.6	3	5.9	9.0	18.9
LP2	4.5	4	5.2	9.0	20.4
RGP1	4.6	3	4.8	11.3	24.8
RGP2	4.0	6	8.8	19.5	39.9

DISCUSSION

Isolation of PSB

PSBs were found in almost all types of soils, although their number varies depending upon the soil and climatic conditions. A considerably higher concentration of phosphate solubilizing bacteria was commonly found in the rhizosphere in comparison with non-rhizosphere soil.[16] The PSB are ubiquitous with variation in forms and population in different soils. Several bacteria species, in association with plant rhizosphere, were capable of increasing availability of phosphorus to plant either by mineralization of organic phosphate or by solubilization of inorganic phosphate.[17] A considerably

higher concentration of phosphate solubilizing bacteria was commonly found in the rhizosphere in comparison with non rhizosphere soil. The soil bacteria belonging to the genera *Pseudomonas* and *Bacillus* and fungi were more common in organic agriculture.[18] The phosphate solubilizing bacteria belongs to the genus *Bacillus* spp, *Citrobacter* spp, *Shigella* spp, *Klebsiella* spp, from sea-grass rhizosphere soil.[19] Likewise,[20] bacterial spp. such as *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus atrophaeus*, *Paenibacillus macerans*, *Vibrio proteolyticus*, *Xanthobacter agilis*, *Enterobacter aerogenes*, *Enterobacter taylorae*, *Enterobacter asburiae*, *Kluyvera cryocrescens*, *Pseudomonas stutzeri* and *Chryseomonas luteola* from mangrove soil. Population of PSB depends on different soil properties and cultural activities.[21] Larger populations of PSB were found in agricultural and range land soils.[22]

The rhizosphere was generally considered to be a narrow zone of soil subject to the influence of living roots, where root exudates stimulate or inhibit microbial populations and their activities of PSB.[23] Among the rhizosphere abundant bacterial genera *Azotobacter*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Microbacterium*, *Pseudomonas*, *Rhizobium* and *Serratia* were most promising PSB.[24]

Identification of PSB

The bacterial cultures morphological, cultural and physiological and biochemical characteristics using the manual of microbiological methods and identified the organism *Bacillus* sp., using Bergey's manual of Determinative Bacteriology.[25] The phosphate solubilizing microorganisms such as *Pseudomonas* a*eruginosa*, *P. cepacia*, *P. fluoresence* and *P. putida* from therhizosphere of wheat and *Bacillus licheniformis*, *B. mycoides*, *B. megaterium* from the rhizosphere of paddy.[26] The morphological and biochemical characters of phosphobacteria were found to be gram negative with rod shaped and non motile characteristics. The organism showed positive results for indole production, methyl red and catalase test.[27]

PSB strains were in rod shape, Gram positive and motile in nature.[28] They were positive to catalase, acid from glucose, casein hydrolysis, gelatin hydrolysis, starch hydrolysis, citrate utilization and nitrate reduction and negative to anaerobic growth, gas from glucose, VP test, indole production and growth with lysozyme. *Pseudomonas* sp. was Gram negative and motile and growth was strictly aerobic and spores were absent. *Pseudomonas* 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. In carbohydrate fermentation showed positive result for fructose and glucose where as negative for lactose and dextrose. The PSB isolates had the morphological features like colourless colonies which did not produce pigment, cells were gram negative, rod shaped and on the basis of biochemical reactions it was found to be *Pseudomonas fluorescens*. Upon gram staining, it was found as gram negative characteristics. Isolate produced slimy, white colonies with irregular margins, cells were gram positive and on the basis of biochemical reactions this isolate was identified as *Bacillus megaterium*.[29]

Based on the biochemical tests, the PSB strains were identified up to species level. The results of various biochemical tests for ten PSB isolates were differed. Among ten PSB isolates, 6 strains (*i.e*.) CP1, TP1, TP2, CTP1, BP1 and BP2 were identified as *Bacillus megaterium*, 2 strains as *Pseudomonas putida* and CP2, CTP2 as *Pseudomonas fluorescens*. *P. putida* were rod shaped, 0.5 - 0.8 × 1.0 - 4.0 µm in size. They were Gram negative, motile and their growth was strictly aerobic and spores were absent in all pseudomonads. They were positive to oxidase, catalase, and arginine dihydrolase, acid from glucose and growth on citrate agar and negative to growth at 41ºC, gelatin hydrolysis, starch hydrolysis and denitrification. *P. fluorescens* were rod shaped and 0.5 - 1.0 × 1.5 - 5.0 µm in size. They were Gram negative, motile and growth was strictly aerobic and not producing spores. On King's B medium they produced fluorescein, a water soluble pigment. Cells were positive to oxidase, catalase, lipase activity, arginine dihydrolase, gelatin hydrolysis, acid from glucose, urease and negative to growth at 41ºC and starch hydrolysis.[30]

Mechanism of phosphate solubilization

The phosphate solubilization by the PSB was accompanied with pH reduction of the culture medium. Maximum pH reduction was 2.8, 1.2 and 0.5 units for *Pseudomonas fluorescens*, *Bacillus megaterium* and *Azospirillum lipoferum* strain, when compared to control pH of 6.8.[31] Phosphorus solubilizing activity was determined by the ability of microbes to release metabolites such as organic acids, which through their hydroxyl and carboxyl groups chelate the cation bound to phosphate, the latter being converted to soluble forms.[32] Inorganic P was solubilized by the action of organic and inorganic acids secreted by PSB in which hydroxyl and carboxyl groups of acids chelate cations (Al, Fe, and Ca) and decrease the pH in basic soils. The lowering in pH of the medium suggested that the secretion of organic acids by the P-solubilizing microorganisms[33] *via* direct oxidation pathway that occured on the outer face of the cytoplasmic membrane.[34] When P was applied to soil it get interact with other metallic elements such as Fe, Al and Ca ions which makes the P unavailable to plants through the formation of ferrous phosphate, aluminium phosphate, calcium phosphate *etc*. and the release of organic acids by PSM leads the chelation reaction and because of this the bound P to other metallic elements get freed and becomes available to plants.

The mechanism of phosphate solubilization by PSB may involve the release of low molecular weight of organic acids, which chelate phosphate bound cations to convert P into soluble forms.[35] Highly efficient PSB have been shown to utilize the direct oxidation glucose pathway to produce gluconic and 2-ketogluconic acids. Microorganisms and their interactions in soil play a critical role in mediating the distribution of P between the available pool in soil solution and the total soil P through solubilization, mineralization and immobilization reactions of sparingly available forms of inorganic and organic soil P. Mechanism of phosphate solublizing includes production of organic acids and production of acid phosphatase.[37,36]

The high alkaline phosphatase enzyme produced by PSB and were responsible for organic solubilizion of insoluble phosphate.[38] However, phosphatase was not directly act on inorganic phosphate solubilization, though phosphatase activity may participate in lowering the pH of the culture medium by the dephosphorylating action and the production of acids.[39] The ability of PSB strains to quantitatively dissolve phosphate and pH of the liquid media was also measured spectrophotometrically. Decrease pH and increase soluble phosphate indicated that the isolates having ability to produce organic acids.[40,41,42] Those strains were able to produce more organic acids or having an effective phosphatase enzyme by which it solubilize maximum amount of phosphate.

Organic acids were found to be responsible for tricalcium phosphate and rock phosphate solubilization.[43] Addition of EDTA was found to increase solubilization of tricalcium phosphate. Organic acids were also found to mediate the release of phosphorus bound to iron oxides.[44] P-solubilizing microbes excrete inorganic acids, organic acids and phosphatase to dissolve phosphorus.[45] However, the mechanisms of P-solubilization not only differ among microorganisms but also dependent on the applied P sources.[46] Phosphate solubilization of most organic phosphorous compounds is carried out by means of phosphatase enzymes. The presence of a significant amount of phosphatase activity in soil has been reported[47]

Important levels of microbial phosphatase activity have been detected in different types of soils.[48,49] In fact, the major source of phosphatase activity in soil is considered to be of microbial origin.[50] In particular, phosphatase activity is substantially increased in the rhizosphere.[51] Phosphatase activity was considered as main contributor towards increased phosphorus and its availability. P-solubilizing activity was determined by the microbial biochemical ability to produce and release organic acids, which through their carboxylic groups chelate the cations, bound to phosphate converting them into the soluble forms.[52] A strain of *Burkholderia cepacia*, commercially used as biofertilizer which display significant mineral phosphate solubilization and moderate phosphatase activity, also improve the yield of tomato, potato, onion, banana, coffee *etc*.[53] 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.[38] Phosphate solubilization by PSB was the action of phosphatase enzyme. The liberation of phosphorus from organic phosphate compounds was mainly due to the action enzyme esterase type. PSB produced the phosphatase enzyme which solubilized the phosphate in the aquatic environment.[54] Acid phosphatase promoted the hydrolysis of the synthetic organophosphatic substrates like p-nitrophenyl phosphate.[55]

CONCLUSION

The population dynamics of PSB was varied in the rhizosphere soils of selected crop plants. The isolated PSB strains were characterized under *in vitro.* All the PSBs isolates were able to solubilize the insoluble phosphate source (Tricalcium phosphate (TCP) and Rock phosphate (RP)) and the solubilizing capacity was varied with strains. PSB strains were able to produce organic acids and phosphatase enzyme and the production ability varied with strain to strain. These variations reflected in the phosphate solubilization.

ACKNOWLEDGEMENT

The authors are thankful to the Management and the Principal of Ayya Nadar Janaki Ammal College, Sivakasi, Tamil Nadu for providing laboratory facilities and financial assistance to carry out this research work.

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