Annals of Plant and Soil Research 22(4): 437-441 (2020)
https://doi.org/10.47815/apsr.2020.10018

Isolation, identification and characterization of phosphate solubilizing bacteria in soils of coal mines landfills of Chhattisgarh

NELSON XESS* AND SHWETA SAO

Microbiology Department, Dr. C.V. Raman University, Kota, Bilaspur, Chhattisgarh, India

Received: July, 2020; Revised accepted: August, 2020

ABSTRACT

Phosphate solubilizing bacteria have the ability to solubilize the insoluble phosphates and to improve the quality of soil health and fertility. Efficacy of phosphate solubilizing microorganisms has been identified on the basis of kinetics and phosphorus accumulation. In this study, twenty eight soil samples were collected from different coal mines landfills of Chhattisgarh and occurrence of phosphate solubilizing bacteria (PSB) was isolated, purified and identified. In addition, the phosphate solubilizing capacity of bacteria based on the formation of visible or halo zone on Pikovskaya agar plates (PVK) and broth having tricalcium phosphate (TCP) and rock phosphate (RP) as a phosphate source was estimated at Dr. C.V. Raman University, Kota, Bilaspur (Chhattisgarh). The result revealed dominance of Pseudomonas syringae as major phosphate solubilizers, along with Bacillus subtillis followed by Pantoea dispersa, Bacillus circulans. Use of these PSB as bioinoculants increased the available P in soil to the extent of 194 and 246 µg/ml of P. the soluble P in case of rock phosphate was less than tricalcium phosphate. The maximum solubilization was observed after 15 days follow by a decrease in amount of soluble P.

Keywords: Phosphate solubilizing bacteria, rock phosphate, tricalcium phosphate, coal minerals.

INTRODUCTION

Phosphorus is the major essential macronutrient of plants and its deficiency is a severe constraint to crop production. Phosphorus is known to involve many functions in plant growth and metabolism. Though soils may have a large reservoir of total phosphorus the amount available to plants is usually but very small proportion of the total phosphorus (Majumdar and Chakraboty 2015). Phosphorus is abundant both in organic and inorganic forms. There are various types of rhizobacteria that can solubilize this fixed form of P and make it available to plants. Such organisms are called Phosphate Solubilizing bacteria. Several phosphate solubilizing bacteria are now known to convert the insoluble form of phosphorus to soluble form through the secretion of chelating organic acid or protons and thus lowering the pH (Richardson *et al.* 2009). PSBs, particularly those belonging to the genera *Pseudomonas* and *Bacillus* possess the ability to convert the insoluble phosphate into a soluble form (Fankem *et al.*2006). Plant takes phosphate in the form of soluble orthophosphate ions ($H_2PO_4^-$ and HPO_4^{2-}). Phosphate rocks should provide a cheap source of phosphorus fertilizer for agricultural use. Inoculation of phosphate solubilizing bacteria with rock phosphate may be considered a better means to overcome the low solubility problems of rock phosphate (Jaleel Basha *et al.* 2018). The unmanaged excess of phosphate application is known to cause environmental problems. The use of such a natural resource constitutes an economic, environmentally friendly, and efficient way of fertilizing crops. In the present study, different strains of PSB using biochemical tests were isolated and identified and investigate highly efficient solubilizers of TCP and RP.

MATERIALS AND METHODS

Total 28 soil samples were randomly collected from different coal mines landfills of Korea, Korba and Surajpur districts, S.E.C.L. (South Eastern Coalfields Limited) Chhattisgarh, India and stored at 4^oC in the laboratory, Department of Life science, University of Dr. C.V Raman University, Kota, Bilaspur for further study. These soil samples were serially diluted using sterile water blanks and plated on the Pikovskaya Agar medium (Marra *et al.*2012). The plates were incubated at 37^0C for 2-4 days. After incubation, the phosphate solubilizing microorganisms were selected based on the zone of clearing around the colonies. The isolated phosphate solubilizing bacteria were purified by repeated culturing and maintained on

Nutrient Agar slants at 4^0C. The most bacterial isolates were identified using standard biochemical tests (Poonguzhali *et al.* 2008).

Screening of PSB: Pure culture of phosphate solubilizing bacteria was spot inoculated on Pikovskaya agar plates and incubated at 37°C. For this Pikovskaya agar plates were formally divided into two parts. 10µl suspension of two days grown culture of bacteria isolates was used to inoculate the center of each half part of the petriplate. The halo zone of phosphate solubilization around growth was recorded (in mm) after every 24 hours of incubation. The colonies forming more than 5.0 mm zone of solubilization were selected as efficient strains (Hamadali *et al.* 2008).

Estimation of phosphate solubilization of tricalcium phosphate: Pikovskaya broth (100 ml) was dispensed in 250 ml conical flasks and TCP (109 mg equivalent 50 mg P_2O_5) was added as a phosphorus source. The flasks were inoculated with bacterial isolates and allowed to grow for different periods of time at 37°C. The soluble phosphorus was determined by the method given by Chen *et al.* (2006). The pH of culture filtrates was also measured.

RESULTS AND DISCUSSION

Isolation and Enumeration of PSB

The Pikovskaya agar plate was inoculated with the suspensions such as 10^{-2} and 10^{-4}, showed more colonies, and no bacterial colonies showed in 10^{-5} dilution (Table 1).Dunfield and Germida, (2001) also supported that the initial isolation of phosphate solubilizers is usually made by using a medium suspended with insoluble phosphates such as tricalcium phosphates. These findings were also supported by Keneni *et al.* (2010). From these soil samples, total 425 bacterial strains were isolated on Pikovskaya agar plates by serial dilution method at 10^{-4} dilution. Out of these 425 bacterial isolates, only 43 bacterial isolates were observed to be formed a halo zone surrounding the colonies that were the 10% of total bacterial population isolated (Table 2). Mohan and Menon (2015) reported the same results in their study.

Table 1: Total rhizobacterial and phosphate solubilizing bacteria present in different coal mines landfills area

District	Coalfields	Rhizobacterial					Phosphate solubilizingbacteria				
		Dilution					Dilution				
		10^{-2}	*10^{-3}*	*10^{-4}*	*10^{-5}*	*Cfu/g of soil*	*10^{-2}*	*10^{-3}*	*10^{-4}*	*10^{-5}*	*Cfu/g of soil*
Korea, Chhattisgarh	Chirmiri Area	>300	192	87	7	87×10^5	49	20	9	0	9×10^5
	Baikunthpur Area	>300	178	81	6	81×10^5	43	22	9	0	9×10^5
Korba, Chhattisgarh	Kusmunda	291	172	48	3	48×10^5	21	9	4	0	4×10^5
	Dipka	>300	184	42	5	42×10^5	47	22	7	0	7×10^5
	Gevra	>300	127	41	3	41×10^5	38	16	5	0	5×10^5
Surajpur, Chhattisgarh	Bhatgaon	286	124	55	4	55×10^5	24	13	4	0	4×10^5
	Bishrampur	>300	203	71	5	71×10^5	39	14	5	0	5×10^5

Table 2: Population density andqualitative screeningof phosphate solubilizing bacteria in different coal mines landfills area

District	**Coalfields**	**Population density of Rhizobacteria 10^5/g of soil**	**Population of PSB 10^5/g of soil**	**No. of less efficient bacteria (>5 mm zone of solubilization)**	**No. of efficient bacteria (<5mm zone of solubilization)**
Korea, Chhattisgarh	Chirmiri	87	9	7	2
	Baikunthpur	81	9	8	1
Korba, Chhattisgarh	Kusmunda	48	4	4	0
	Dipka	42	7	4	3
	Gevra	41	5	3	2
Surajpur, Chhattisgarh	Bhatgaon	55	4	3	1
	Bishrampur	71	5	3	2
Total		425	43	32	11

Qualitative screening of PSB

Qualitative screening of phosphate solubilizing bacterial isolates revealed variations in phosphate solubilization efficiency of selected isolates. Data showed that the section of efficient phosphate solubilizing bacterial isolated on qualitative basis.The data (table 3) indicated the value of the diameter of the colony(C), diameter of halo zone (H), diameter of colony+ halo zone (Z) and the ratio Z/C of different isolates obtained on PKV agar plates showed more than 5mm zone of phosphate solubilization. The use of the ratio Z/C helps to evaluate the activity of a given microorganism. The phosphate solubilization activity of these selected isolates on PVK agar plates ranged from 1.33 to 2.55 (Table 3).

Table 3: Phosphate solubilization activity of selected bacterial isolates

Isolates	**Diameter of colony (C)**	**Diameter of halo zone (H)**	**Diameter of (colony+ halo zone) (Z)**	**Solubilization activity (Z/C)**
PSB 1	11	5	16	1.45
PSB 2	9	11	20	2.22
PSB 3	14	6	20	1.42
PSB 4	12	4	16	1.33
PSB 5	9	12	21	2.33
PSB 6	14	5	19	1.35
PSB 7	9	6	15	1.66
PSB 8	4	5	9	2.25
PSB 9	9	14	23	2.55
PSB 10	7	5	12	1.71
PSB 11	12	5	17	1.41

Solubilization of tri-calcium phosphate and rock phosphate

The quantitative solubilization and screening were done in Pikovskaya broth having tricalcium phosphate (TCP) and rock phosphate (RP) as a phosphate source. The quantitative tests to assay the relative efficiency of the phosphate solubilizing microorganisms are based on the lowering of pH, owing to the production of organic acids into the surrounding medium. Out of 11 bacteria isolates, only 4 isolates were found to be highly efficient solubilizers of TCP which showed maximum solubilization in fifteen days. These four isolates were PSB 2, PSB 5, PSB 8 and PSB 9. These isolates showed maximum solubilization between 194 and 246 µg/ml of P and PSB 9 showed maximum solubilization at 15th day was 246 µg/ml of P (Table 4).

Table 4: Soluble phosphate and pH reduction of the Pikovskaya media (TCP equivalent to 50 mg P_2O_5 inoculated with 11 bacterial isolates after different time intervals

Bacterial Isolates	Soluble P (µg/ml) After 5 Days	After 5 Days pH	Soluble P (µg/ml) After 10 days	After 10 days pH	Soluble P (µg/ml) After 15 days	After 15 days pH	Soluble P (µg/ml) After 20 days	After 20 days pH
PSB1	32.32±3	5.45±0.4	39.47±0.4	5.21±0.5	31.21±0.3	5.17±0.3	31.76±0.5	5.15±0.2
PSB2	64.75±3	5.10±0.3	184.56±2	4.55±0.7	194.21±4	4.32±0.3	181.51±5	4.22±0.5
PSB3	39.05±2	5.12±0.1	98.00±4	4.86±0.4	81.22±5	4.78±0.3	87.36±5	4.67±0.4
PSB4	32.38±4	5.82±0.4	62.12±5	5.09±0.5	76.29±5	4.78±0.2	72.81±3	4.48±0.3
PSB5	58.10±3	6.50±0.4	180.18±5	6.21±0.4	235.00±3	4.48±0.2	229.22±5	4.43±0.2
PSB6	45.76±2	6.64±0.4	70.12±3	5.75±0.3	71.75±4	5.46±0.4	65.43±5	5.42±0.4
PSB7	38.12±4	4.46±0.2	80.21±2	4.33±0.4	97.71±5	4.20±0.3	102.08±3	4.13±0.3
PSB8	79.38±5	5.23±0.6	181.40±5	4.22±0.4	203.12±3	4.33±0.45	192.12±5	4.15±0.4
PSB9	52.38±0	6.14±0.2	204.22±3	4.82±0.2	246.00±2	4.10±0.4	237.10±4	4.24±0.2
PSB10	52.48±4	5.32±0.4	60.00±2	4.78±0.2	91.74±4	4.12±0.4	106.21±4	4.04±0.2
PSB11	28.54±4	6.75±0.4	54.32±2	6.64±0.4	98.72±2	6.54±0.3	91.45±3	6.47±0.2

Highly efficient solubilizers of TCP which showed maximum solubilization of four isolates PSB 2, PSB 5, PSB 8 and PSB 9 were tested for bacterial solubilization of the rock phosphate (149.6 mg equivalent 50 mg P_2O_5 in 100 ml PKV broth). The soluble P in case of RP was less than TCP in the isolates. The maximum solubilization was observed after fifteen days and after that the soluble P decreased. These four isolates showed significantly highly efficient solubilization of rock phosphate (20 to 34 µg/ml of P) and pH range was 4.1 to 4.4 (Table 5).

Table 5: Soluble phosphate and pH reduction of the Pikovskaya media (rock phosphate equivalent to 50 mg P_2O_5) inoculated with 4 bacterial isolates after different time intervals

Bacterial Isolates	Soluble P (µg/ml) After 5 days	After 5 days pH	Soluble P (µg/ml) After 10 days	After 10 days pH	Soluble P (µg/ml) After 15 days	After 15 days pH	Soluble P (µg/ml) After 20 days	After 20 days pH
PSB2	7.89±2	6.14±0.5	14.46±3	5.45±0.2	21.05±2	4.10±0.4	18.18±4	4.24±0.2
PSB5	12.18±2	5.42±0.4	19.46±4	5.12±0.4	30.85±3	4.48±0.4	29.02±2	4.18±0.2
PSB8	8.56±3	6.10.±0.5	12.21±5	5.22±0.4	20.12±5	4.11±0.2	19.18±5	4.05±0.5
PSB9	14.26±3	5.85±0.3	21.72±4	5.23±0.6	34.44±4	4.44±0.4	32.19±2	4.22±0.5

Based on the biochemical tests, the PSB isolates, named PSB 2, PSB 5, PSB 8 and PSB 9 were identified as *Bacillus subtillis, Bacillus circulans,Pantoea dispersa* and *Pseudomonas syringae* (Table 6). The PSB strains were identified up to species level. Kumar *et al.* (2012) studied the bacterial cultures morphological, cultural and physiological, and biochemical characteristics and identified the organism *Bacillus* sp., Richardson (2001) found that the predominant soil bacteria involved in phosphate solubilization include *Bacillus* and *Pseudomonas*.

Table 6: Morphological, physiological, and biochemical characteristics of phosphate solubilizing bacteria strains

Characteristics	PSB strains Code No.			
	PSB2	*PSB5*	*PSB8*	*PSB9*
Gram staining	Gram positive	Gram positive	Gram negative	Gram negative
Shape of cell	Rod	Short Rod	Rod	Short Rod
Spore	Sporulating	Sporulating	Non-Sporulating	Non-Sporulating
Pigments	No pigment	No pigment	Yellow	Creamy white
Motility	Motile	Motile	Motile	Motile
Catalase	+	+	+	+
Oxidase	-	-	-	+
Methyl red	-	-	+	-
Voges Proskauer	+	-	+	-
Indole	-	-	-	-
H_2S production	+	-	-	-
Citrate Utilization	+	-	+	+
Starch hydrolysis	+	+	-	-
Casein hydrolysis	+	+	-	+
Gelatin hydrolysis	+	+	+	+
Oxidative fermentation	-	-	+	+
Anaerobic growth	-	-	-	-
Urease	-	-	-	+

Result showed *Pseudomonas syringae* has greater phosphatesolubilization efficiency along with *Bacillus subtillis* followed by *Pantoea dispersa* and *Bacillus circulans.*

REFERENCES

Chen, Y.P, Rekha, P.D, Arun, A.B, Shen, F.T, Lai, W.A, and Young, C.C. (2006) Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities. *Applied Soil Ecology* **34**(1):33–41.

Dunfield, K.E, and Germida, J.J. (2001) Diversity of bacterial communities in the rhizosphere and root interior of field-grown genetically modified Brassica napus. FEMS *Microbiology Ecology* **38**(1): 1-9.

Fankem, H., D. Nwaga, A. Deubel, L. Dieng, W. Merbach & F. X. Etoa. (2006) Occurrence and functioning of phosphate solubilizing microorganisms from oil palm tree (*Elaeis guineensis*) rhizosphere in Cameroon. *African Journal of Biotechnology* **5**(24): 2450-2460**.**

Hamadali, H, Hafidi, M, Virolle, M.J, and Ouhdouch, Y (2008) Rock phosphate solubilizing actinomycetes: screening for plant growth promoting activities. *World Journal of Microbiology and Biotechnology* **24**(11): 2565–2575.

Jaleel Basha,I. Singh, A.K., Engrala Ao, and Gupta R.C. (2018) Evaluation of Udaipur rock phosphate as phosphorus fertilizer in soyabean (*Glycine max* L. Merrill) grown on acid upland soils of Nagaland. *Journal of the Indian Society of Soil Science* **66**(1): 83-88

Keneni, A., Assefa, F., Prabu, P.C. (2010) Isolation of phosphate solubilizing bacteria from the rhizosphere of faba bean of Ethiopia and their abilities on solubilizing insoluble phosphates.*Journal of Agricultural Science and Technology* **12**: 79-89.

Kumar, P, Dubey R.C, and Maheshwari, D.K. (2012) Bacillus strains isolated from rhizosphere showed plant growth promoting and antagonistic activity against phytopathogens. *Microbiological Research* **167**(8): 493–499.

Majumdar, S., and Chakraboty, U. (2015) Phosphate solubilizing rhizospheric *Pantoea agglomerans* Acti-3 promotes growth in jute plants. *World Journal of Agricultural Sciences* **11**(6): 401-410.

Marra, L.M, Soares, CRFSS, de Oliveira, S.M, Ferreira, P.A.A.A, Soares, B.L, Carvalho, R.F, Lima, J.M, and Moreira, F.M. (2012) Biological nitrogen fixation and phosphate solubilization by bacteria isolated from tropical soils. *Plant and Soil* **357**(1): 289–307.

Mohan, V., Menon, S. (2015) Diversity Status of Beneficial Microflora in Saline Soils of Tamil Nadu and Pudhucherry in Southern India. *Journal of Academia and Industrial Research* **3**(8):384-392.

Poonguzhali, S., Madhaiyan, M., Sa T, (2008) Isolation and identification of phosphate solubilizing bacteria from chinese cabbage and their effect on growth and phosphorus utilization of plants. *Journal of Microbiology and Biotechnology* **18**(4):773–777.

Richardson, A. E. (2001) Prospects for using soil microorganisms to improve the acquisition of phosphorus by plants. *Australian Journal of Plant Physiol*ogy **28**(9): 897-906.

Richardson, A.E., Barea, J.M., McNeill, A.M. and Prigent-Combaret, C. (2009) Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms.*Plant and Soil* **321**(1-2): 305-339.