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ISOLATION, SCREENING AND IDENTIFICATION OF PHOSPHATE SOLUBILIZING BACTERIA FROM DIFFERENT REGIONS OF VISAKHAPATNAM AND ARAKU VALLEY

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ABSTRACT

A study was undertaken to investigate the occurrence of phosphate solubilizing bacteria (PSB) from soil samples collected across different regions of Visakhapatnam & Araku valley, and to evaluate the potential of phosphate solubilizing bacteria (PSB). Eighteen soil samples were collected randomly to isolate phosphate solubilizing bacteria (PSB) by using plate assay method. A novel defined microbiological growth medium, National Botanical Research Institute's phosphate growth medium (NBRIP), and Pikovskaya medium (PVK), was developed for screening phosphate solubilizing microorganisms. The concentration of solubilized phosphate was determined using Fiske & Subbarow method. The improvement of soil fertility is one of the most common strategies to increase agricultural production. Upon screening, 12 isolates showed varying levels of phosphate solubilizing activity in both agar plate and broth assays using PVK and National Botanical Research Institute's phosphate media. The isolated PSB microorganism were identified as *Bacillus* sp, *Neisseria* sp, *Klebsiella* sp, *Enterobacter sp*, *Pseudomonas* sp., *Proteus* sp. Among these isolates *Bacillus* and *Pseudomonas* species served as efficient phosphate solubilizers and could be considered as an appropriate substitute for chemical phosphorous fertilizer in organic and sustainable agricultural systems. Soil fertility management by bio- fertilizers (PSB) are one of the basic components of sustainable agriculture.

Key words: Phosphate solubilizers; Phosphate solubilizing bacteria (PSB), Soil fertility,

INTRODUCTION

Phosphate solubilizing bacteria (PSB) are the bacteria that possess the capability to change the insoluble form of phosphorus into soluble one. Phosphorus is one the most essential element for plant growth second only to nitrogen in requirement for plants. Phosphorus plays a significant role in physiological and biochemical plant activities. But, due to different chemical reactions there is limited availability of this nutrient for plants especially in arid and semi-arid

soils. Most of the essential plant nutrients remain in insoluble form in soil [1]. Approximately 95–99% of soil phosphorous is present in the form of insoluble phosphates and cannot be utilized by the plants [27]. A greater portion of inorganic phosphates applied to soil as fertilizer is rapidly immobilized after application therefore; it becomes unavailable to plant Thus, the insoluble and fixed form of phosphorous is released in order to increase the soil phosphorous availability [2]. Seed or soil inoculation with phosphate-solubilizing bacteria is known to improve solubilization of fixed soil phosphorus and applied phosphates resulting in higher crop yields [30].

The best suitable pH for phosphorous uptake by plants is 6.5 this was indicated by [16]. Phosphorus plays a significant role in physiological and biochemical plant activities like photosynthesis, transformation of sugar to starch and transporting of the genetic traits [22]. Phosphorous also causes early ripening in plants, decreasing grain moisture, improving crop quality and is the most sensitive nutrient to soil pH. The advantage of feeding the plants with phosphorus creates deeper and more abundant roots [23]. PSB have been used to improve rock P value because they convert insoluble rock P into soluble forms available for plant growth [18]. This conversion is through acidification, chelation and exchange reactions [8]. Solubilization of inorganic insoluble phosphate by microorganisms leads to the production of organic acids and chelating oxo acids from sugars. Several mechanisms like lowering of pH by acid production, ion chelation and exchange reactions in the growth environment have been reported to play a role in phosphate solubilization by Phosphate solubilizing microorganisms (PSMs) [11]. PSMs play an important role in supplementing phosphorus to the plants, allowing a sustainable use of phosphate fertilizers. PSB has good ability to facilitate phosphorus uptake and auxine hormone secretion which effectively enhances the plant growth. PSB isolated from soils produce IAA, GA3and cytokinin like substance, which ultimately enhance the plant metabolism. Production of IAA varies greately among different crops and is also inflenced by culture conditions,growth stage and availability of substrate(S).

Bio-fertilizers (phosphate solubilizing bacteria) are, considered among the most effective plant assistants to supply phosphorus at a favorable level. These fertilizers are produced on the basis of selection of beneficial soil microorganisms which have the highest efficiency to enhance plant growth by providing nutrients in a readily absorbable form. Application of inoculants provided from these microorganisms enhances an abundant population of active and effective microorganisms to the root activity zone which increases plant ability to uptake more nutrients [15]. The growth of Phosphate solubilizing bacteria depends on cultural activities and different soil properties such as physical and chemical properties, organic matter, and soil phosphorus content [14].

Rock phosphate is a non-detrital sedimentary rock which contains high amounts of phosphate bearing minerals.Rock phosphate is generally in granular or crystalline form The two main sources for phosphate are guano, formed from bird droppings, and rocks containing concentrations of the calcium phosphate mineral, apatite. Nitrogen in rock originates as organically bound nitrogen associated with sediment, or in thermal waters representing a mixture of sedimentary, mantle, and meteoric source of nitrogen. Nitrate deposits accumulated in arid and semi-arid regions are also large potential pools. Nitrogen in rock has a potentially significant impact on localized nitrogen cycles. Elevated nitrogen concentrations in water and soil have been attributed to weathering of bedrock nitrogen Rock materials and inoculants when mixed thoroughly with the soil, ensure the

solubilization effect of rock nutrient by plant growth promoting rhizobacteria PGPRs [10].

Among the soil bacterial communities *Pseudomonas, Bacillus, Enterobacter and Klebseilla* strains could be referred to as the most important strains. In particular, *Pseudomonas* sp. has high phosphorous uptake efficiency. This bacterium has a notified importance as a biological fertilizer because of the ecotype diversity of this species and its tolerance in some environmental stresses [4].

One of the most important means to achieve the goals of sustainable agriculture is to extent the application of biofertilizers as these biofertilizers are considered as the most favorable natural compounds to enhance the micro-organism activities in the soil. In order to reach this goal, the chemical fertilizers and pesticides should be moderately used and in the mean time the soil organic matter content should be increased.

In the present the isolation and characterization of phosphate solubilizing bacteria. (Qualitative estimation),Determination of solubilized phosphate concentration. (Quantitative estimation) and evaluation of the potential of PSB as fertilizer were undertaken.

2. MATERIALS AND METHODS:

2.1Collection of sample:

Surface (0-15cm) soil samples were collected randomly from different regions. All samples were kept in plastic bags and transported to the laboratory and stored at 4ºC prior to be analyzed. These samples were air-dried and ground to pass through 2mm sieve before the chemical analyses. The samples were analyzed for pH, soil chemistry and texture.

2.2Microbial count

Microbial population was estimated by plate count method [21,28]. Ten grams soil was suspended in 90 ml sterile distilled water in Erlenmeyer flask and mixed thoroughly for 30 minutes using a mechanical shaker at 110 rpm. Then 1ml an aliquot transferred with sterile pipettes to 9 ml sterile distilled water in test tube. This suspension was stir for 10second. A subsequent serial dilution was prepared as above to 10^{-7}. From each serial dilution, 0.2 ml of aliquot was transferred to sterile petriplate and over poured and dispersed swirling with agar media.

2.3. Isolation and characterization of phosphate solubilizing bacteria.(Qualitative estimation)-

Bacteria representative of the predominant morphological types present on the plates were selected at random and purified on minimal medium [5]. National Botanical Research Institute's phosphate growth medium (NBRIP), and Pikovskaya [19] medium (PVK), was developed for isolation and identification of phosphate solubilizing Microorganisms.The composition of the media was shown in the **Table 1 & 2.**

Phosphate solubilisation test was conducted qualitatively by plating the bacteria in Pikovaskaya agar media (Table1) and NBRIP media (Table 2). PSB was grown on Pikovskaya agar media [24,9], diluted in 1 l distilled water. The pH of the media was adjusted to 7.0 before autoclaving. Bacterial strains were tested by plate assay using PVK and NBRIP. Four strains per plate were stabbed in triplicate using sterile toothpicks. The halo and colony diameters were measured after 14 days of the incubation of plates at 28ºC. Colonies of PSB were detected by clear zones of solubilization around them. The isolates were identified following Bergey's manual for bacteriology methods systematic [13].

2.4 Determination of solubilized phosphate concentration. (Quantitative estimation)

Quantitative estimation of solubilized phosphate broth was carried out using Erlenmeyer flasks (150 ml) containing 10 ml of medium inoculated in triplicate with the bacterial strain (100µl inoculums with approximately 1-2x10^{9}cfu ml^{-1}). Sterile uninoculated medium served as control. The flasks were incubated for 2 days at 30ºC in a

refrigerated incubator shaker at 180 rpm. The cultures were harvested by centrifugation at 10000 rpm for 10 min, using centrifuge. Phosphate in culture supernatant was estimated.

The concentration of solubilized phosphate in culture supernatant was determined to observe the kinetics of solubilization of each isolate using Fiske and Subbarow method.

2.5. ***Evaluating the potential of PSB as fertilizer.***

The potential of phosphate solubilizing bacteria (PSB) is evaluated by inoculating the PSB in nutrient limited soil planted with *Capsicum annum* (pepper) *and Phaseolus vulgaris* (white bean) and its impact on mineral uptake and growth of plant (pepper and bean) is evaluated by co-inoculating phosphate solubilizing bacteria with nitrogen fixing bacteria. Phospahate solubilizing bacteria are isolated using NBRIP and PVK media, which was developed for screening phosphate solubilizing microorganisms and Yeast mannitol agar medium, are used for isolating Nitrifying bacteria. These nitrifying bacteria can be grown on selective media containing adequate amounts of ammonia and nitrates.The Phosphate solubilizing bacteria and Nitrifying bacteria were cultured in their individual medium respectively, and then incubated on an orbital shaker at 150 rpm for 48 h at 27°C. The cells in cultured bacterial broth were collected by centrifugation at 2.822 × g for 15 min at 4°C and washed with sterilized tap water. The pelleted cells were resuspended with sterilized tap water and then the cells were adjusted to about 108 cells/ml, based on optical density OD620 = 0.08 [3], and 1 ml of inoculums was applied into each seedling.

Experiment for studying the effect of the bacterial strain on plant growth, P and N uptake of pepper and bean plants was conducted in pots (17 cm diameter and 15 cm deep) layered with plastic bags and containing 2.0 kg of sterilized soil. The soil was sterilized by dry autoclaving at 20 psi for 120 min. the experiment was established with 5 treatments i.e, Soil without rock P and N materials or without bacteria inoculation which serves as a control,Soil with PSB (Pseudomonas flourescens) inoculated, Soil with Nitrifying bacteria (Nitrobacter) inoculated , Soil inoculated with both PSB, Nitrifying bacteria and Soil with only N and P rock materials.

Rock materials and inoculants were mixed thoroughly with the soil in a plastic pot to ensure the solubilization effect of rock nutrient by PGPRs. (plant growth promoting rhizobacteria). Seeds of pepper and bean were surface-sterilized in 2% sodium hypochlorite for 3 min and then rinsed 5 times with distilled water [3]. The seeds were germinated and grown in sterilized vermiculite in trays. At 7 days after sowing for pepper and bean, one seedling was transplanted into each pot. At three days after transplanting, depending on the treatment, the healthy seedling was inoculated with 1 ml of inoculums containing around 108 cells. The temperature around the potted plant was maintained at 30 ± 2°C with a relative humidity of 65% and a 16 hrs photoperiod created by using supplemental lighting from high pressure sodium lamps. All plants were harvested 20 days after transplanting.

Growth of the plant in each pot was observed and the potential of PSB and its effect of co-inoculation with nitrifying bacteria on mineral uptake and growth of pepper and bean plant was known, along with Increasing N uptake by the plants with inoculation with *Bacillus.*, a genus which fixes atmospheric nitrogen in symbiosis with legume, Therefore, *Bacillus* strain used in this study might have the capacity to fix atmospheric nitrogen. It is also known that P availability in soils is important for the uptake of N from soils and its utilization in plant [14], this can be studied in the present experiment.

RESULTS AND DISCUSSION:

Totally, 36 isolates obtained from 18 soil samples collected randomly, and the samples were tested for phosphate solubilizing property, of these 22

were found to be PSB. Upon screening, 12 isolates showed varying levels of phosphate solubilizing activity in both agar plate and broth assays using NBRIP and PVK medium. Identified isolates from 18 regions of Visakhapatnam and Aruku valley are showed in the Table3.

The most dominant phosphate solubilizing bacteria found were aerobic and of which, some are spore forming bacteria. Identification of this group showed that *Bacillus* sp. was the most predominant PSB was found in all of soils tested, followed by *B. cereus, B. subtilis* and *B. megatherium* were in soil samples numbers 2, 3,5,12,13,and 15 respectively as shown in the Table 3. Other PSB involved were Klebsiella sp., K. aerogenes, K. pneumoniae, *Proteus* sp., *P.mirabilis, P vulgaris, P.inconstans, Enterobacter sp., E. aerogens, and Pseudomonas* sp. Sporeformers were well known to resist adverse conditions such as high temperature and dryness this was observed by Taha et al [26]. Thus the important PSB can overcome such unfavorable conditions.

The result analysis of the concentration of solubilized phosphate which was determined using Fiske C.H. & Subbarow Y [6] method and also indirect measurement of phosphate solubilization by plate assay were the halo and colony diameters of the incubated agar plates were reported in the Table 4.From the data tabulated the bacterial isolates Proteus vulgaris, Pseudomonas fluorescens, Bacillus cereus, B.megaterium, B.subtilis, Proteus inconstans, P.mallei, K.pneumoniae and E.aerogens showed high phosphate solubilization activity. From the results it also indicates that the most efficient PSB can be screened using NBRIP broth assay.

The graphical representation of the data indicates that the phosphate solubilizing bacteria showed high phosphate solubilization activity by plate assay and broth assay using NBRIP media when compared to PVK media as shown in Figure1 & 2.

Result analysis for evaluating the potential of PSB as fertilizer showed that

Addition of rock materials into the soil did not significantly increase available P or N and the plant growth (figure 3b), was slow when compared to other and single inoculation of PSB or Nitrifying bacteria (NFB) resulted in a higher mineral availability in which PSB strain was a more potent solubilizer for rock P than NFB strain (figure 3c and 3d). When applied all together, PSB, NFB and rock P and N resulted in the highest availability of P and N in soils, and caused an increase of P and N (figure 3e)as compared to untreated control (without bacterial inoculum and without rock material fertilizer)(figure 3a).

N uptake in the plant increased with inoculation with *Bacillus*, a genus which fixes atmospheric nitrogen in symbiosis with legume [28], this was observed by the increase in the growth of the plant. (Figure 3f)Therefore, *Bacillus* strain used in this study might have the capacity to fix atmospheric nitrogen. It is also known that P availability in soils is important for the uptake of N from soils and its utilization in plant [14]. The highest availability of P and N in soils was seen in the pot (figure 3d), where PSB, NFB and rock P and N applied all together this resulted in, increase of P and N (figure 3e) as compared to untreated control and rest of the planted pots (figure 3a, 3b, 3c, 3d).

CONCLUSION:

The present investigation was carried out to study the occurrence of PSB. The isolated microbes were identified, screened and characterized. Most of the bacteria were isolated from soil samples with pH values close to 7 and 8. There is a close relationship between the phosphate solubilizing activity and low pH levels in the growth medium. This suggests that phosphate solubilization could be the result of organic acids released from bacterial metabolism, as reported in literature. The bacterial isolates Pseudomonas sp, *Bacillus* sp,

Klebsiella sp, *Enterobacter sp, Proteus* sp were identified as PSB. *Bacillus* strain used in this study might have the capacity to fix atmospheric nitrogen. It is also known that P availability in soils is important for the uptake of N from soils and its utilization in plant. Therefore the application of these phosphate solubilizing bacteria (PSB) could be considered as an appropriate substitute for chemical phosphorous fertilizer in organic and sustainable agricultural systems. [20,7].

Different soil nutrient status and vegetation type in the investigated sites resulted in the different bacterial population and bacterial type. The difference was caused by releasing organic and inorganic root exudates that can be used by surrounding organism. Jha et al. [12] and Setiadi [25] found that biological activity and composition of soil microbes are generally affected by many factors including physico-chemical properties of soil, temperature and vegetation. .

The discovery of mutual relationship between plants and phosphate solubilizing bacteria (PSB), in which bacteria provide soluble phosphate and plants supply root borne carbon compounds (mainly sugars), that can be metabolized for bacterial growth, hence for biofertilization the use of PSB improves the crop yield.

In short, results from all these and other experiments suggest that co-inoculation of PGPR with different beneficial properties should be the future trends of bio-fertilizer application for sustainable crop production. Soil fertility management by bio- fertilizers (PSB) are one of the basic components of sustainable agriculture.

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S.No	Components	PVK gm/L	NBRIP gm/L
1.	Glucose	10	10
2.	$Ca_3(PO_4)_2$	5	5
3.	$(NH_4)_2SO_4$	0.5	0.1
4.	NaCl	0.2	0.2
5.	$MgSO_47H2O$	0.1	0.25
6.	KCl	0.2	0.2
7.	Yeast extract	0.5	-
8.	$MnSO_47H2O$	0.002	-
9.	$FeSO_47H2O$	0.002	-
10.	Agar	15	15

Table1: Composition of Pikovaskaya media (PVK) & National Botanical Research Institute's Phosphate growth media (NBRIP)

Soil Sample	Isolated Phosphate Solubilizing bacteria
Sample -01	*Proteus vulgaris,Branhamella catarrhalis*
Sample -02	*Bacillus megateriym*
Sample -03	*Bacillus cereus*
Sample 04	*Proteus mirabilis; Moraxella bovis; Micrococcus varians; Staphylococcus aureus*
Sample -05	*B. catarhalis; M bovis; B.subtilis*
Sample -06	*Corynebacterium kutscheri*
Sample -07	*Streptococcus species;Proteus inconstans*
Sample -08	*Corynebacterium Xerosis; Neisseria mucosa; Klebsiella aerogens*
Sample -09	*Citrobacter freundii*
Sample -10	*N. mucosa; Streptococcus species*
Sample -11	*Klebsiella pneumonia; N. mucosa; Streptococcus species*
Sample -12	*M. bovis; C. Xerosis*
Sample -13	*Staphylococcus aureus; Streptococcus species*
Sample -14	*Micrococcus luteus; Lactobacillus fermenti*
Sample -15	*Corynebacterium kutscheri; Pseudomonas mallei*
Sample -16	*Bacillus megatarium*
Sample -17	*Staphyloccus saprophyticus; Pseudomonas flourescens*
Sample -18	*Bacillus subtillus; M.bovis; Enterbacter aerogens.*

Table 2: List of isolated Pphosphate Solubilizing Bacteria from 18 regions of Visakhapatnam & Aruku valley.

Bacteria	Agar (halo size(mm))		Broth (µg ml^{-1}P solubilized)	
	PVK	NBRIP	PVK	NBRIP
Proteus vulgaris	3	2	18	27
Bacillus cereus	6	3	14	26
Proteus mirabilis	8	-	20	28
Micrococcus varians	-	2	14	26
Staphyloccus aureus	4	-	11	18
Proteus inconstans	-	5	18	30
Klebsiella pneumonia	8	4	20	28
Pseudomonas mallei	4	-	16	35
Bacillus megaterium	-	-	14	32
Bacillus subtilis	6	2	12	35
Enterobacter aerogens	5	3	25	34
Pseudomonas fluorescens	8	3	20	36

Table 3 : The Phosphate solubilization by bacterial isolates in agar and broth using Pikovskaya medium (PVK) and National Botanical Research Institute's phosphate growth medium (NBRIP) medium.

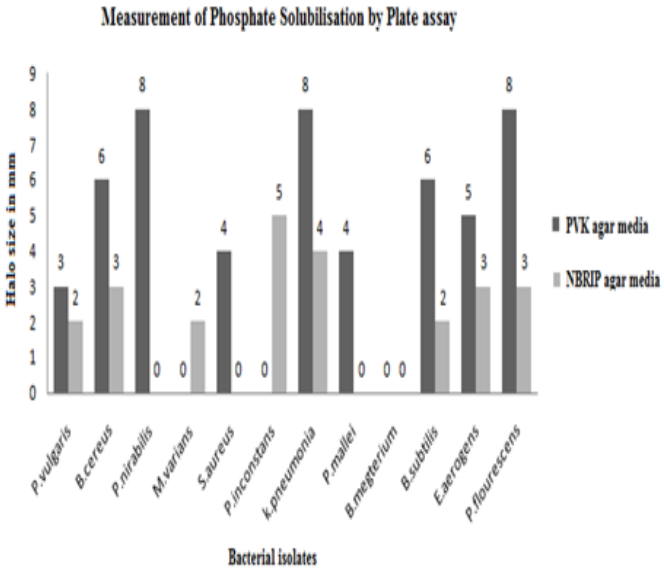

Figure 1: Measurement of Phosphate solubilizaton by plate assay.

The graphical representation of the data indicates that the phosphate solubilizing bacteria showed high phosphate solubilization activity by plate assay using NBRIP agar media when compared to PVK agar media

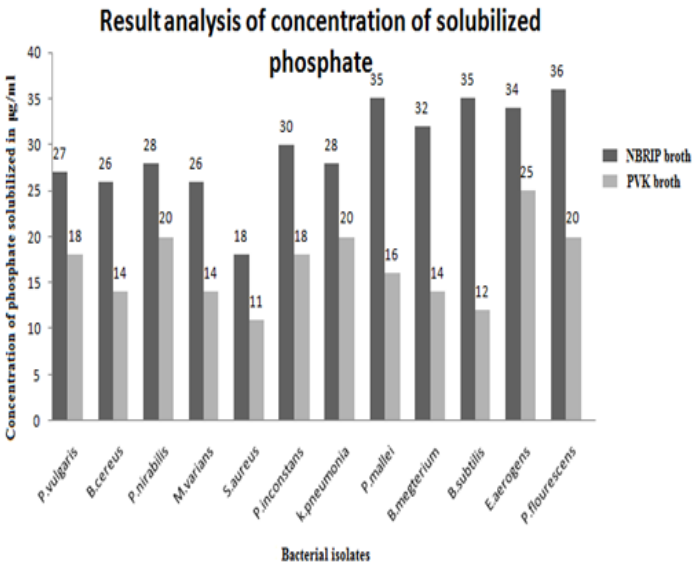

Figure 2: Result Analysis of Solubilized Phosphate using broth assay. The graphical representation of the data also indicates that the most of the phosphate solubilising bacteria showed high concentration of solubilized phosphate using NBRIP broth assay when compared to PVK broth.

Fig3f	Fig3a	Fig3b	Fig3c	Fig3d	Fig3e

Fig3 : Evaluating the potential of PSB as fertilizer inoculated in nutrient limited soil planted with *Capsicum annum(pepper) and Phaseolus vulgaris(bean)at 26°C temperature* .

Figure 3a Plant potted with pepper containing soil without Phosphorous and Nitrogen materials or without bacterial inoculation served as control showed limited groeth.

Figure 3b Plant potted with pepper containing soil only with Phosphorous and Nitrogen materials showed slow growth when compared to other.

Figure 3c Plant potted with pepper containing soil inoculated only with PSB (Pseudomonas flourescens) resulted in the highest availability of phosphorous and plant showed increase in growth compared to **Fig 3a& 3b**

Figure 3d Plant potted with pepper containing soil inoculated only with Nitrifying bacteria (Nitrobacter) also showed increase in the growth of the plant compared to **Fig 3a, 3b,** but less growth when compared to **Fig 3c.**

Figure 3e Plant potted with pepper containing soil inoculated with PSB and Nitrifying bacteria) resulted in the highest availability of phosphorous and nitrogen hence the plant showed significant increase in its growth compared to **Fig 3a, 3b, 3c, & 3d.**

Figure 3f Plant potted with bean containing soil inoculated with Bacillus, a genus which fixes atmospheric nitrogen in symbiosis with legume, this was observed ny the growth of the plant. Therefore Bacillus strain used in the study might have the capacity to fix atmospheric nitrogen.