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STUDIES ON ISOLATION AND CHARACTERIZATION OF PHOSPHATE SOLUBALIZING BACTERIA ISOLATED FROM WHEAT PLANT RHIZOSPHERE (*Triticum aestivum)* OF PIPRI REGION OF WARDHA DISTRICT, MAHARASHTRA

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ABSTRACT

Plant growth promoting rhizobacteria (PGPR) plays an important role in the sustainable agriculture industry. The demand of increasing crop production with significant reduction of chemical fertilizers and pesticides is a big challenge nowadays. Our study aims to isolate and characterize phosphate solublizing bacteria (PSB) isolated from the rhizosphere soil of plant *Triticum aestivum* (wheat) from the agricultural field of pipri region of Wardha district, Maharashtra. The soil samples were collected and analyzed for isolation of PSB in the month of December 2017. Total ten PSB were isolated and studied for their PGPR traits like production of Ammonia, Indole acetic acid production (IAA) and hydrogen cyanide (HCN). Out of ten isolates, four isolates showed the highest ability to solublize the insoluble phosphate. These four PSB strains were morphologically and biochemically characterized. Out of these four isolates PSB1, PSB2, PSB3 were identified as *Pseudomonas* sp. and PSB4 as *Bacillus* sp. The potential of isolates to solublize the insoluble phosphate was studied qualitatively and quantitatively. Phosphate solublizing index of isolates was determined as 31.09, 29, 29 and 15 with efficiency of phosphate solublization as 360 µgml^{-1}, 210 µgml^{-1}, 165 µgml^{-1}, 145 µgml^{-1} for PSB1, PSB2, PSB3 and PSB4 respectively. The isolates were also tested for their potential of heavy metal tolerance and found to tolerate different types of heavy metals. PSB1 has metal tolerance 75, 50, 50, PSB2 with 100, 75,100, PSB3 with 100, 75, 50 and PSB4 has 100, 75,100 for Zn, Co and Hg respectively. The isolates of present study hold a promise as effective PGPR for wheat crop.

(Key words: Plant growth promoting rhizobacteria (PGPR), *Triticum aestivum,* wheat rhizosphere)

INTRODUCTION

Phosphorus is a most important plant macronutrient that plays a significant role in plant metabolism. It is an essential macronutrient for plant growth and development since it is important component of biological molecules, such as DNA, RNA, ATP, and phospholipids. It affects root development, stalk and stem strength, crop maturity, and nitrogen fixation in legumes (Khan *et al.*, 2009). Out of added phosphorus fertilizer only 10-20 % is available for the plants. The rest remains in the soil as insoluble phosphate in the form of rock phosphate and tricalcium phosphate. Phosphorus in soils can exist in both organic (Po) and inorganic (Pi) forms; the inorganic forms of phosphorus have been calculated to account for 35 – 70% of total P in soil (Harrison, 1987). It is estimated that about 98% of indian soils contain insufficient amounts of available phosphorus (Padmavathi and Usha, 2012). Phosphate solubilising Bacteria (PSB) are a group of beneficial bacteria capable of hydrolysing organic and inorganic phosphorus from insoluble compounds. The metabolic activities of microorganisms solubilize phosphate from insoluble calcium, iron and aluminium phosphates. These biochemical changes that take place in the soil prove that microorganisms perform various essential functions that contribute to the productivity of soil.

The plant growth promoting Rhizobacteria (PGPR) is a group of bacteria capable of colonizing actively plant roots system and improving their growth and yield (Wu *et al.*, 2005). The term "PGPR" was firstly proposed by Kloepper *et al.* (1980) and was used especially for the fluorescent *Pseudomonas* involved in the improvement of plant growth and biological control of pathogens. Nowadays, this expression is used to refer to all bacteria living in the rhizosphere (around roots), improving plant growth by one or several mechanisms (Haghighi *et al.*, 2011). A large range of species belonging to the genus *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Klebsiella*, *Enterobacter*, *Alcaligenes*, *Arthrobacter*, *Burkholderia*, *Bacillus*, and *Serratia* were reported to be PGPR (Saharan and Nehra,2011). According to Nakkeeran *et al.* (2005) an ideal PGPR should possess high rhizosphere competence,

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enhance plant growth capabilities, have a broad spectrum of action, be safe for the environment, be compatible with other rhizobacteria, and be tolerant to heat, UV radiation, and oxidizing agent. Considering the factors discussed above, the need for a better PGPR biofertilizer to complement skyrocketing agricultural food production.

Utilization of PGPR is dependent on its survival in soil, environmental factors, the interaction ability with indigenous microflora of soil and the compatibility with the crop on which it is inoculated (Martinez *et al*., 2010). All rhizobacteria does not possess the same mechanisms of action so the modes of action of PGPR are diverse (Dey *et al*., 2004; Choudhary *et al*., 2011). These disadvantages limit the application of PGPR. Therefore, the competition between synthetic chemical fertilizers and PGPR as a biofertilizer is deemed redundant in the face of the global agricultural productivity needed to feed the booming world's population, which is predicted to escalate to 8 billion people by 2025 and 9 billion by 2050.

In this context, the aim of our study was to isolate and identify the potential of PGPR from wheat (most cultivated and consumed cereal in Wardha region of Maharashtra) rhizosphere. The objective of this study is to propose biological fertilizers based on native PGPR for farmers for increasing wheat production.

MATERIALS AND METHODS

Soil sample collection

The roots of wheat (*Triticum aestivum*) plants and the immediate surrounding soil were collected from agricultural field of Pipri region of Wardha, Maharashtra in the month of December 2017 in sterile sample bag. The plant roots and soil samples were transported to the laboratory, and portions were immediately plated for rhizobacteria isolation. The remainder of each sample was preserved at 4 ^{O}C for further analysis.

Isolation of phosphate-solubilizing bacteria from wheat rhizosphere soil samples

Approximately 2 gms of soil sample was scraped from the roots of each sample and deposited into sterile tubes containing 2 ml of sterile distilled water. Each test tube was vortexed thoroughly and a series of 10-fold dilutions was prepared down to 10^{-9}. 100 ìl from each dilution was plated on Pikovskaya (PVK) media agar plates. Pikovskaya medium: Glucose (10 gl^{-1}),Yeast extract(0.5 gl^{-1}),$MgSO_4$-$7H_2O$(0.1 gl^{-1}), KCl(0.2 gl^{-1}), Ammonium sulphate (0.5 gl^{-1}), $MnSO_4$- (trace), $FeSO_4$- (trace), tricalcium phosphate (5.0 gl^{-1}), Agar (20 gl^{-1}), Distilled water(1L), pH – 7 (adjusted by 0.1 N KOH) and autoclaved at 121^{0}C for 20 min. The insoluble Ca_3 $(PO_4)_2$ was washed with DI water and centrifuged to remove soluble phosphate contaminants. The supernatant was discarded, and the wet Ca_3 $(PO_4)_2$ was dried by using a vacuum-flask apparatus. The colonies that produced clearing zones in the PVK agar plates were isolated. Individual colonies from the isolation were then respotted onto new PVK plates for better analysis of clearing zone formation. All plates were incubated at 30 ^{0}C for up to 7 days. (Sharon *et al.*, 2016).

Identification of Rhizobacteria

The identification of isolated Rhizobacteria consisted firstly in macroscopic (colony morphology, pigmentation, etc.) and microscopic (Grams reaction, motility, cell shape, etc.) observations. This first identification was followed by several biochemical and enzymatic tests. The performed tests are production of oxidase, catalase, indole, voges-proskaur test, methyl red test, hydrogen sulfide, citrate utilization, fermentation of glucose and lactose, manitol and sucrose.

Qualitative determination of phosphate solubilazing efficiency

The qualitative determination of the isolated PSB to solubilize tricalcium phosphate on National Botanical Research Institute's phosphate growth medium (NBRIP), was determined in terms of solubilization index (SI). NBRIP agar contained, Glucose (10gl^{-1}), tricalcium phosphate (5gl^{-1}), Magnesium chloride hexahydrate (5gl^{-1}), Magnesium sulphate heptahydrate (0.25 gl^{-1}), Potassium chloride (0.2 gl^{-1}), Ammonium sulfate (0.1gl^{-1}), Double distilled water (1L), Agar (20 gl^{-1}).

Phosphate solubilization index was calculated by measuring the colony diameter and the halo zone diameter using the following formula.

Phosphate Solubilization Index (SI) = (colony diameter +clearing zone) / colony diameter (Karpagam and Nagalakshmi, 2014).

Quantitative assay of phosphate solubilizing activity

Quantitative estimation of solubilized phosphate by PSB was done by using Fiske-Subbarow method. 1 ml of bacterial culture was inoculated in 100 ml NBRIP broth and flasks were incubated in rotary shaker at 30^{0}C and 200 rpm for 11 days. After each day samples were regularly taken for phosphate estimation. 0.5ml of supernatant was mixed with 1 ml of 2.5 M sulphuric acid and 2.5% ammonium molybdate. To the above mixture 1ml of reducing agent (0.2 g of 1-amino -2- napthol-4-sulfonic acid and 1.2 g of sodium sulfite in 100 ml distilled water) was added and incubated at room temperature for 10 minutes and the absorbance was read at 650 nm using potassium dihydrogen phosphate as standard. (Kapri and Tewari ,2010).

Characterization of isolates for PGPR traits

Assay for Indole acetic acid (IAA) Production

IAA production was detected by the method of Brick *et al*. (1991). All four isolates were freshly grown on their respective growth medium amended with tryptophan with varying concentration which ranged from 0 to 500 µg ml^{-1} at 30^{0}C for 48 hours. Fully grown cultures were centrifuged at 8000 rpm for 10 minutes and was assayed for

quantitative measurement of IAA. 2 ml of supernatant was mixed with two drops of orthophosphoric acid and 4 ml of salkowski reagent (1 ml of 0.5M $FeCl_3$ in 50 ml of 35% $HClO_4$). Formation of pink colour indicates IAA production. Optical density was taken at 540 nm. Concentration of IAA produced by cultures was measured with the help of standard graph of IAA (Hi-media) obtained in the range of 10–100 μgml^{-1}.

Production of Ammonia

All the four isolates were tested for the production of ammonia. Freshly grown isolates were inoculated in 10 ml peptone broth and incubated for 24 hrs at 30 ^{0}C. The test was performed by adding 0.5 ml of Nesslers reagent in each tube. Development of brown to yellow colour was a positive test for ammonia production (Cappuccino and Sherman, 1992)

Siderophore Production

Siderophore production was determined on Chrome azurol S agar (CAS) by the method given by Schwyn and Neilands (1987). CAS agar plate divided into equal sectors and spot inoculated with test organisms and incubated at 28 – 30^{0}C. Development of orange halos around the colonies indicates positive siderophore production.

Hydrogen Cynide (HCN) production

Qualitative HCN determination was carried out by Lorck (1948) method modified by Alstrom and Burns (1989). Isolates were cultured on Nutrient agar medium supplemented with glycine (4 / 4 gl-1). The production of HCN was detected after 48 hrs by using Whatman filter paper no. 1 soaked in 2 % sodium carbonate and 0.5% picric acid fixed to the underside of the petri-dish lids which were sealed with parafilm before incubation at 28 to 30 ^{0}C. A change from yellow to orange, red, brown, or reddish brown was recorded as an indication of weak, moderate, or strongly cyanogenic potential, respectively.

Production of enzyme Catalase

Freshly grown cultures were mixed with appropriate amount of 3% H_2O_2 to demonstrate production of enzyme catalase (Bumunang and Babalola, 2014).

Heavy metal tolerance

The isolates were tested for their heavy metal tolerance by broth dilution method. Freshly prepared nutrient broth was amended with Zn, Co and Hg at various concentrations ranging from 25 to 100 μgml^{-1} and was inoculated with fresh cultures of isolates (Wani *et al.*, 2007).

RESULTS AND DISCUSSION

Isolation and biochemical characterization

In the present study, total four isolates were found to have phosphate solublizing ability. These isolates were characterized morphologically and biochemically. On the basis of cultural, morphological and biochemical characteristics as described in Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994) the isolates PSB1, PSB2 and PSB 3 were identified as *Pseudomonas* sp. and isolate PSB 4 as *Bacillus* sp. respectively (Table 1).

Plant growth promoting traits of test isolates

Screening results of PGPR traits are depicted in table 2. IAA production was shown in all the isolates of *Pseudomonas* sp. and *Bacillus sp.* All isolates were found to be negative for siderophore production, whereas positive for ammonia and hydrogen cynide production (Table 2).

Qualitative assay of phosphate solublization

Qualitative assay of phosphate solublization was studied in terms of phosphate solublizing index (Table 3 and 4) and the isolates PSB 1, PSB 2, PSB 3 and PSB 4 showed the phosphate solubilizing index values of 4.09, 3.8, 3.8 and 2.4 respectively after 6 days of incubation on NBRIP medium.

Quantitative assay the phosphate solublization

Quantitative assay of phosphate solubalization was estimated by using Fiske subbarow method. In this assay after 11 days of incubation in liquid medium the isolates PSB 1, PSB 2, PSB 3 and PSB 4 showed 360 ($\mu g\ ml^{-1}$), 210 ($\mu g\ ml^{-1}$), 165 ($\mu g\ ml^{-1}$) and 145 ($\mu g\ ml^{-1}$) of phosphate solublization efficiency (Fig 1).

Heavy metal tolerance

The isolates were studied for their heavy metal tolerance potential (Table 5) and showed heavy metal tolerance ranging from 50 to 100 $\mu g\ ml^{-1}$. highest tolerance is shown by PSB 2 and PSB 4 (100 $\mu g\ ml^{-1}$) towards Zn and Hg while lowest by PSB 1 (50 $\mu g\ ml^{-1}$) towards Co and Hg. A varying level of resistance to heavy metals among the PGPR (*Bacillus* and *Pseudomonas*) have also been reported (Niranjan-Raj *et al.*, 2004) as seen in present study. Several studies have also established a correlation between bacterial antibiotic resistance and metal tolerance (Wani *et al.*, 2007).

Table 1. Biochemical characteristics of PSB

Tests	Phosphate solubilizing bacterial isolates			
	PSB 1	PSB 2	PSB 3	PSB 4
Grams Reaction	-	-	-	+
Motility	+	+	+	+
Glucose fermentation	+	-	-	-
Sucrose fermentation	+	-	-	-
Lactose fermentation	+	-	-	-
Mannitol fermentation	+	+	+	+
H_2S Production	+	+	+	+
Indole test	+	+	+	-
Methyl Red test	-	-	-	-
Vogues- Proskauer test	+	+	+	+
Citrate utilization test	+	+	+	+

Table 2. PGPR traits of PSB

Sr. No.	Tests	PSB1	PSB2	PSB3	PSB4
1	Catalase +	-	+	-	
2	IAA production	+	+	+	-
3	Ammonia production	+	+	+	+
4	HCN production	+	+	+	+
5	Siderophore production	-	-	-	-

Table3. Colony and Halozone Diameter of PSB

Days	Day 1		Day 2		Day 3		Day 4		Day 5		Day 6	
PSB isolates	**Holo-zone diameter (mm)**	**Colony-zone diameter (mm)**	**Holo-zone diameter (mm)**	**Colony-zone diameter (mm)**	**Holo-zone diameter (mm)**	**Colony-zone diameter (mm)**	**Holo-zone diameter (mm)**	**Colony-zone diameter (mm)**	**Holo-zone diameter (mm)**	**Colony-zone diameter (mm)**	**Holo-zone diameter (mm)**	**Colony-zone diameter (mm)**
PSB 1	12 mm	8 mm	20 mm	10 mm	28 mm	10 mm	29 mm	10mm	30 mm	10mm	34 mm	11 mm
PSB 2	13 mm	8 mm	15 mm	8 mm	20 mm	9 mm	23 mm	9mm	27 mm	9mm	28 mm	10mm
PSB 3	14 mm	9 mm	16 mm	10mm	20 mm	9 mm	22 mm	9mm	27 mm	9mm	28 mm	10mm
PSB 4	10 mm	8 mm	11 mm	8 mm	12 mm	9 mm	13 mm	8mm	13 mm	8mm	14 mm	10 mm

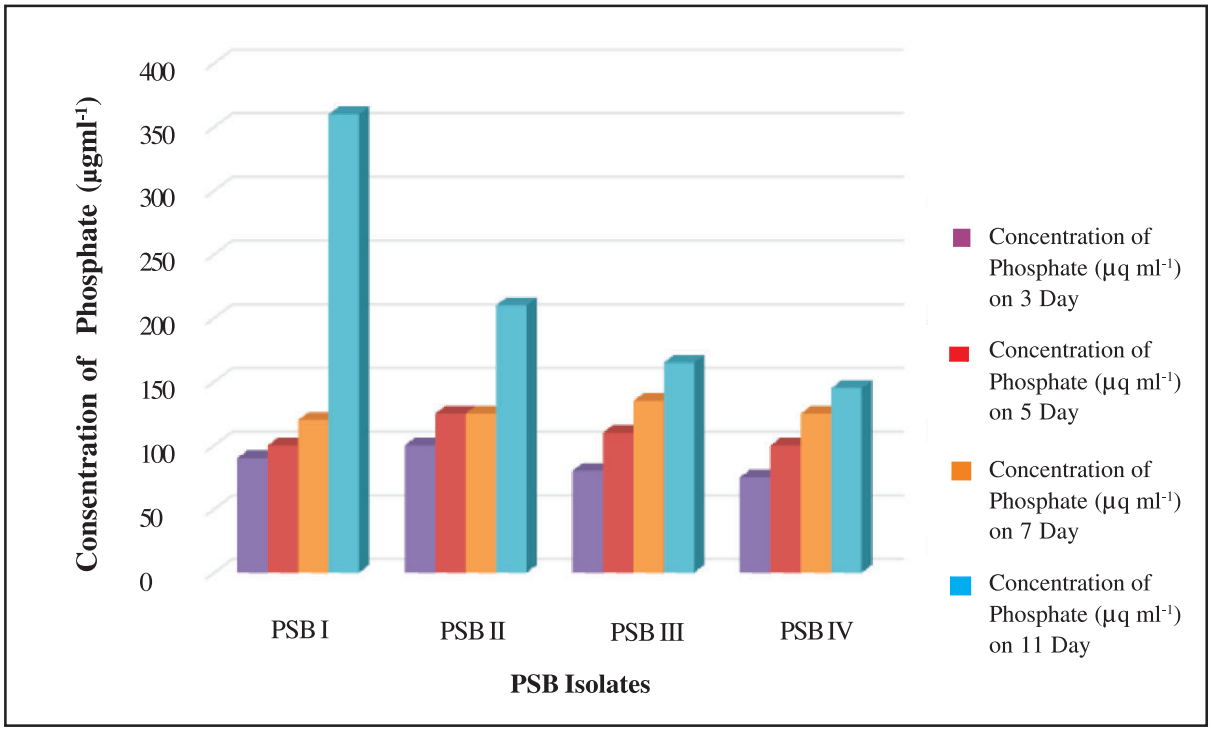
Table 4. Phosphate solubilizing index of PSB on NBRIP agar

Days	1 Day	2 Day	3 Day	4 Day	5 Day	6 Day
PSB 1	2.50	3.00	3.80	3.90	4.00	4.09
PSB 2	2.62	2.50	3.22	3.55	4.00	3.80
PSB 3	2.55	2.60	3.22	3.44	4.00	3.80
PSB 4	2.25	2.37	2.33	2.62	2.62	2.40

Table 5. Heavy metal tolerance of PSB (μg^{-1} ml)

Sr.No.	Isolates	Zn	Co	Hg
1	PSB1	75	50	50
2	PSB2	100	75	100
3	PSB3	100	75	50
4	PSB4	100	75	100

Fig :1 Quantitative estimation of solubilized phosphate by PSB



In this study, screenings for phosphate-solubilizing bacteria from samples collected from a farm of pipri region of Wardha district, Maharashtra was done. Data revealed that bacteria closely associated with roots of wheat (*Triticum aestivum*) plants and the immediate surrounding soil solubilized significantly more $Ca_3(PO_4)_2$. We isolated a bacterium, *Pseudomonas* sp. and *Bacillus* sp., with efficient phosphate solubilisation capabilities from an organic garden. This strain was found to be a very effective biofertilizer. All strains produced indole acetic acid,hydrogen cyanide and ammonia (100%). The hydrogen cyanide is part of powerful antifungal compounds produced by PGPR and involved in pathogens biological control (Haas and Defago, 2005).

One of our strains had the highest level of phosphate solubilization from the insoluble tricalcium complex in liquid culture ever reported to our knowledge and its rate is (360 mg l^{-1}). This strain was characterized and designated *Pseudomonas* sp. and its rate of $Ca_3 (PO_4)_2$ solubilization significantly exceeded that of other studied microorganisms, such as *Pantoea agglomerans* (200 mg l^{-1}) (Son *et al*., 2006), *Pseudomonas fluorescens* (184 mg l^{-1}) (Katiyar and Goel, 2003), *Pseudomonas putida* (247 mg l^{-1}) (Pandey *et al*., 2006), *Bacillus megaterium* (140 mg l^{-1}) (El-Komy, 2005), and *Enterobacter cloacae* (127 mg l^{-1}) (Chung *et al*.,2005).

It should be noted that such comparisons should be evaluated with the caveat that the potential variability of media and growth conditions among different laboratories may have a significant impact. Thus, *Pseudomonas* sp. represents a promising candidate for biofertilizer development in regions with high calcium-bound phosphate levels.

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