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Screening of Potential Heavy Metal Tolerant Phosphate Solubilizing Bacteria from the Rhizosphere of Castor (*Ricinus communis*) Fields

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Abstract: Phosphorous is one of the most vital micronutrients required for the growth and development of plants. Phosphate solubilizing bacteria enhances P availability in soil through dissolving inorganic P pool. 17 phosphate solubilizing bacterial strains were isolated from the rhizosphere of Castor (*Ricinus communis),* fields of Nalgonda district, Telangana State, India, on NBRIP agar. PSB1, PSB9 & PSB16 showed highest solubilization 370, 408 and 415µg/ml and belongs to the genera *Bacillus*, *Pseudomonas* and *Klebsiella*. 9 efficient PSB strains were further screened for their phosphate solubilization under heavy metal stress. 6 different heavy metals (Cu, Co, Cd, Pb, Ni & Cr) each with 4 concentrations (50, 100, 200 & 400µg/ml) were used. PSB9 showed tolerance to all heavy metals and solubilized the TCP under highest concentration i,e 400µg/ml, except for Ni and Cr where it shows the growth. PSB11 and PSB13 are considered as most vulnerable to heavy metals as it do not show phosphate solubilization under metal stress. The presence of heavy metals above critical concentration not only harmfully affects the human health but also the environment. Hence the results obtained suggest that, the isolated PSB9, a *Pseudomonas* strain can be used as a source to supply phosphorous to the plants in heavy metal contaminated soil.

Keywords: Rhizosphere, Phosphate solubilizing bacteria, *Pseudomonas*, *Bacillus*, Heavy metal Tolerance

INTRODUCTION

P (Phosphorous) is second only to Nitrogen as most essential macro-nutrient by plants[1].Plant absorbs P in soluble form of inorganic phosphate, making up about 0.2% of plants dry weight[2] .The average P content in the soil is approximately 0.05%, very less 0.1 % of the total is available to the plants[3] and a shortage of available P in soil is one of the key chemical factor restricting plant growth and development[4] by influencing various key metabolic processes such as cell division, energy transport, macromolecular biosynthesis, respiration and photosynthesis in plants[5] . The bioavailability of soil inorganic phosphorous in the rhizosphere varies considerably with plant species, nutritional status of soil and ambient soil condition[6].To make it available to the plants, phosphatic fertilizers are applied , but 75-90% of the applied soluble forms of phosphate fertilizers are easily precipitated as aluminum phosphate in acidic soil or calcium phosphate in alkaline soil making phosphorous inaccessible to plants[7].However under such conditions microorganisms offers a biological rescue, some beneficial microorganisms in the soil are found to convert insoluble phosphate into soluble form by the process of acidification, chelation and exchange reactions[8].These reactions normally occurs in rhizosphere[9],where the population of PSM(Phosphate Solubilizing Microorganisms) is more in comparison to non-rhizospheric region. But the population, growth, survival activities and phosphate solubilization of PSMs are greatly influenced by soil physical, chemical and biological stresses[10].Various domains of PSM, generally bacteria and fungi have been reported to solubilize inorganic phosphate compounds to soluble forms[11].Most dominant PSBs (Phosphate Solubilizing Bacteria) belongs to the genera *Bacillus*, *Pseudomonas*, *Rhizobium*, *Mesorhizobium*, *Azotobacter*, *Erwinia*, *Enterobacter*, *Acinetobacter*, *Flavobacter*, *Klebsiella*, *Micrococcus*, [12] *Aerobacter*, *Achromobacte*, *Burkholderia*.[13]

Excessive use of fertilizers, pesticides, industrial activities and mining can result in heavy metal contamination[14] .Accumulation of heavy metals in soil, not only causes severe environmental and human health hazard problem but also affects plant growth and more adversely affects the plant beneficial microorganisms like PSMs and their physiological activities associated with the soil fertility. Therefore isolation of heavy metal tolerant microorganisms which possess plant growth promoting characteristics is of particular importance for the degraded and polluted lands[15]. Hence the present study was aimed to isolate, a potential PSB that can tolerate high concentration of heavy metals, get adapted to stress along with providing soluble forms of phosphate to plants. Such PSM may prove to be an ideal microbe that may support bioremediation.

MATERIALS AND METHODS

Soil samples Collection: Soil samples were collected from the rhizosphere of Castor fields from the Nalgonda district of Telangana State, India. The soil samples were taken within the rhizospheric circumference of 1-10cm radius by 2-10 cm depth and brought to the laboratory under aseptic conditions[16].

Isolation of indigenous rhizospheric bacteria: From each soil sample 10gm were transferred into 250ml of Erlenmeyer flask containing 90ml of sterile saline and serial dilution method was employed[17]. Aliquots of serially diluted soil samples were spread on nutrient agar plates and incubated at 30^0C for 48 hrs. Predominant and morphologically distinct colonies were selected and purified by repeated culturing and maintained on nutrient agar slants at 4^0C[18].

Screening of PSB, using NBRIP medium: Each bacterial isolate were aseptically transferred onto National Botanical Research Institute Phosphate agar media containing (Glucose-10gm,Tri-Calcium Phosphate-5gm,$MgCl_2$-5gm,$MgSO_4$-0.25gm,KCl-0.2gm,$(NH_4)_2SO_4$-0.1gm,Agar-15g/liter)[19] by point inoculation method using sterile needle and incubated at 30^0C for 5 days.[20] Solubilization index was measured by using following formula[21].

SI = (Colony Diameter + Halo zone Diameter) / Colony Diameter

Only the bacterial strains which forms halo around their colonies were selected for quantitative analysis of phosphate solubilization.

Quantitative Estimation of Phosphate solubilization: Phosphate solubilization was done by growing the isolates in National Botanical Research Institute's phosphate growth medium, containing 0.5% TCP. The flask containing 50 ml medium, was inoculated with 1.0 ml bacterial suspension ($3x10^7$cells/ml) and incubated at 30^0C at 200 rpm for 5 days[22].The uninoculated autoclaved medium, incubated under a similar set of condition served as control. At the end of incubation period cell suspension was centrifuged at 10,000 rev min^{-1} for 10 minutes and the P content in the supernatant was spectrophotometrically estimated by the Ames method[23] and pH of the culture medium was measured with a pH meter.

Identification of PSB: Microscopic identification was carried out by Gram Staining. Morphological and biochemical tests were performed as per the Bergey's Manual of Systematic Bacteriology[24].

Phosphate Solubilization under Heavy metal stress: Isolated bacterial strains were assessed for their tolerance to heavy metal by using agar dilution method[25]. Freshly prepared NBRIP agar plates were amended with 6 different heavy metals (Cu, Co, Cd, Pb, Ni & Cr) at various concentrations (50, 100, 200 & 400 µg/ ml). Bacterial isolates were inoculated by point inoculation method, P solubilization and heavy metal tolerance was determined by the appearance of halo zone and bacterial growth after 2 days of incubation at 30^0C[15].

Statistical Analysis: All experiments were carried out in triplicates (n=3), results are expressed as

Mean + SD.

RESULTS AND DISCUSSION

Isolation of PSB from the rhizosphere: Out of 40 bacterial strains isolated from the rhizospheric soil, 17 showed the phosphate solubilization on NBRIP agar medium. Maximum solubilization index value

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was observed in PSB16 (3.44) followed by PSB9 (3.27), PSB1 (3.12). For the isolation of PSB several types of media could be used for example Pikovyskaya agar[26], Sperber medium[27] etc.

Quantitative Estimation of Phosphate Solubilization: All the PSB isolates were further evaluated by broth assay and PSB16 showed highest solubilization (415 µg/ml) after 5 days of incubation followed by PSB9 (408µg/ml) and PSB1 (370 µg/ml). The size of the zone of solubilization on agar and the corresponding phosphate solubilizing activity in broth (µg/ml), measured after 5 days of incubation, were found to be in conformity, similar results were reported by Pandey *et al* [28] (Table -1). Although phosphate solubilizing activity measured in the form of halo zone on agar and estimated using broth culture are not always found to be similar[29].The pH of the broth was found to decline from initial 7±2 to 3.21, lowering of pH coincide with an increase in efficiency of phosphate solubilizing activity[20] . Research work of Vyas *et al* and Vassileva *et al* suggested that decrease in the pH of culture filtrate containing inorganic phosphate is due to the secretion of organic acids by bacteria in the culture media[30,31]. Hence acidification of culture supernatants can be the main mechanism for P solubilization.

Table1: Phosphate solubilizing activities of PSB isolates.

Isolates	Solubilization Index (SI)	P Solubilization µg/ml	Final pH
PSB1	3.12± 0.15	370± 0.57	3.43±0.12
PSB2	2.19 ± 0.12	245±1.52	3.96±0.51
PSB3	1.93 ± 0.11	210± 0.02	4.42±0.32
PSB4	2.64± 1.20	285±0.01	3.49±0.41
PSB5	2.96± 0.20	290±1.15	4.89±0.02
PSB6	2.27± 0.15	220±0.05	4.17±0.59
PSB7	2.3 ± 0.05	238±0.73	4.11±0.68
PSB8	2.8 ± 0.37	296±0.03	4.72±0.02
PSB9	3.27± 0.25	408±0.29	3.33±0.81
PSB10	1.24±1.82	163±1.22	5.42±0.19
PSB11	2.72±0.19	335±0.89	3.84±1.01
PSB12	2.34±0.15	230±2.09	4.17±1.22
PSB13	2.92±0.18	275±1.74	5.36±1.16
PSB14	2.52±0.09	261 ±1.32	4.49±0.48
PSB15	1.58±0.02	196±1.28	4.91±0.26
PSB16	3.44±0.16	415±0.92	3.21±0.76
PSB17	1.46±0.28	183±0.76	5.38±0.93

Values are mean of three replicates; Initial pH= 7±2

Identification of bacterial isolates: Out of 17 PSB isolates, based on phosphate solubilizing potentiality 09 were selected for further experiments. All the bacterial isolates were rod shaped and out of 9 phosphate solubilizers, 5 are Gram negative and 4 are Gram positive. These isolates were further identified by biochemical reactions and belong to the genera *Bacillus, Pseudomonas and Klebsiella*. All these isolates are potent phosphate solubilizers and our results are supported by the work of many other researchers[13, 32].

Table 2: Morphological and Biochemical Characteristics of the Efficient PSB isolates

Characteristics	**PSB1**	**PSB4**	**PSB5**	**PSB8**	**PSB9**	**PSB11**	**PSB13**	**PSB14**	**PSB16**
Grams Nature	+	-	+	-	-	+	+	-	-
Motility	+	+	+	+	+	+	+	+	-
Shape	IR	R	IR	R	R	IR	IR	R	R
Color	white	white	white	yellow	Pale	white	pale	cream	pale
BIOCHEMICAL TEST									
Indole	-	-	-	-	-	-	-	-	-
Methyl Red	-	-	-	-	-	-	-	-	-
Voges-Proskeur	+	-	+	-	-	-	-	-	+
Citrate Utilization	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+
Oxidase	-	+	-	+	+	-	-	+	-
H2S Production	-	+	-	+	+	+	+	+	+
Starch Hydrolysis	+	-	+	-	-	+	+	-	-
Urea Hydrolysis	-	-	-	-	-	-	-	-	+
Gelatin Hydrolysis	+	-	+	+	+	+	+	+	-
Nitrate Reduction	+	+	+	+	+	+	+	+	+
CARBOHYDRATE FERMENTATION									
Dextrose	+	+	+	+	+	+	+	+	+
Lactose	-	-	-	-	-	-	-	-	+
Sucrose	+	+	-	+	+	+	+	+	-
Mannitol	-	-	+	-	-	+	+	-	+
Xylose	-	+	+	+	+	+	+	+	+
Remarks	**B**	**P**	**B**	**P**	**P**	**B**	**B**	**P**	**K**

IR-Irregular; R-Regular, B= *Bacillus*, P=*Pseudomonas*, K=*Klebsiella*

Phosphate Solubilization under Metal Stress: Microorganisms have developed the mechanism to cope with a variety of toxic metals for their survival in the environment enriched with such metals[33].Hence bacterial tolerance to heavy metal is an important consideration when bacteria are to be introduced into soils for enhancing bioremediation of metal contaminated soils. Phosphate solubilizing ability in addition to metal tolerance may prove to be a suitable combination for promoting plant growth in metal contaminated sites[10]. Out of 9 PSB isolates, *Pseudomonas* strain PSB9, proved to be the best isolate among all, as it has tolerated all concentrations of metals and solubilized Tri-calcium phosphate, followed by *Klebsiella* (PSB16) and *Bacillus* (PSB1) (Table 3). These results are in accordance with the work of Monica *et al*[10].*Pseudomonas species*, has the highest potential for dissolving P in soil contaminated with metal. The high potential of the *Pseudomonas species* can be attributed to greater production of extracellular polymer & organic acids[34].PSB11 and PSB13 *Bacillus* strains are most vulnerable to the heavy metals Nickel and Chromium. Zinc and Nickel tolerance by *Rhizobium leguminosarum*, isolated from sewage sludge treated soil was reported by Purchase & Miles[35].

There are a number of studies demonstrating the importance of bacterial inoculation for plant growth and bioremediation in heavy metal polluted environments[36,37].Studies of Halstead suggested that inorganic phosphate solubilization facilitates the uptake of the metals from the soil[38].Hence the isolation of microorganisms possessing efficiency in inorganic phosphate solubilization and tolerance to heavy metal can be useful to speed up the recolonization of the plant rhizosphere in polluted soil[39].

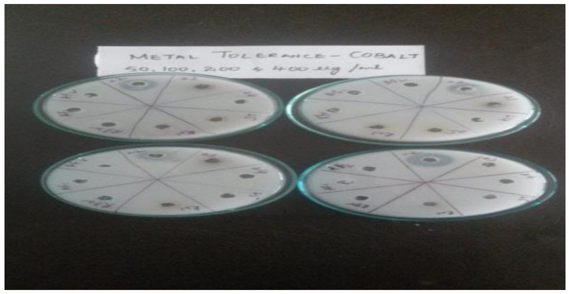

Fig: PSB isolate showing tolerance to Cobalt at different concentrations.

Table3: Heavy metal Tolerance among isolated Phosphate solubilizing bacteria

Metal µg/ml \ Isolates		Phosphate solubilization zone in mm								
		PSB1	**PSB4**	**PSB5**	**PSB8**	**PSB9**	**PSB11**	**PSB13**	**PSB14**	**PSB16**
Copper	**50**	0.6	0.3	0.2	0.4	0.5	+	+	0.1	0.4
	100	0.3	0.1	+	0.3	0.3	+	+	+	0.3
	200	+	+	+	0.2	0.2	-	+	+	0.2
	400	+	+	-	+	0.1	-	+	+	+
Cobalt	**50**	0.6	0.2	0.1	0.1	0.5	+	+	0.3	0.2
	100	0.3	0.1	+	+	0.4	+	+	0.2	0.2
	200	0.3	0.1	+	+	0.3	-	+	+	0.1
	400	0.1	0.1	-	-	0.2	-	-	+	0.1
Cadmium	**50**	1	0.3	+	+	0.6	+	+	0.1	0.4
	100	0.5	0.1	+	+	0.3	-	-	0.1	0.2
	200	0.3	+	-	+	0.2	-	-	+	0.2
	400	0.1	+	-	-	0.1	-	-	+	0.1
Lead	**50**	0.2	0.2	+	-	0.3	+	+	+	0.2
	100	0.1	+	+	-	0.3	-	-	+	0.1
	200	+	+	-	-	0.2	-	-	+	+
	400	+	+	-	-	0.2	-	-	+	+
Nickel	**50**	0.1	0.1	+	+	0.2	-	-	+	0.2
	100	+	+	+	+	0.1	-	-	+	+
	200	+	+	-	-	0.1	-	-	-	+
	400	+	+	-	-	+	-	-	-	+
Chromium	**50**	0.2	0.1	+	+	0.3	-	-	+	0.2
	100	+	-	-	-	0.2	-	-	+	+
	200	+	-	-	-	0.1	-	-	-	+
	400	+	-	-	-	+	-	-	-	+

Tolerance to Heavy metal (+), Susceptible to Heavy metal (-)

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CONCLUSION

Based on the results obtained in this study, we can conclude that isolated bacterial strains PSB1, PSB9 and PSB16 (*Bacillus sp*, *Pseudomonas sp and Klebsiella sp*, respectively) are potential inorganic phosphate solubilizers, as they showed their stable heavy metal tolerant character against 6 different metals with various concentrations. These tolerant strains should be further evaluated for other plant growth promoting activities also under field condition to assess their agricultural and environmental significance.

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