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Phosphate Solubilizing Bacteria and Fungi Isolated from the Salinity Affected Soil and its Growth Promotion on Soybean Plant

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Abstract: The use of phosphate solubilizing bacteria (PSB) as biofertilizer has concurrently increased phosphorous uptake in plants and improved yields in several crop species. A laboratory study was conducted to isolate, identify and characterize the phosphate solubilizing bacteria from salinity affected area of Amravati district. (*Aspergillusniger*, *Penicilliumrugulosum* and *Trichoderma harzanium*) have the more solubilizing ability of inorganic insoluble phosphate than bacteria, i.e., *Bacillus cereus*, *Bacillus megaterium*, *Bacillus subtilis*, *Bacillus polymyxa*, *pseudomonas putida*, *pseudomonas straita*, *Micrococcus lotus* , *Enterobacterclocoue* Hence the application of biofertilizer prepared by above mentioned fungi should be helpful to increase the crop yield in salinity affected soil by solubilizing large concentration of inorganic insoluble phosphate. Application of all isolated culture with lignite showed plant growth promoting activity.

Keywords: Phosphate Solubilizing Bacteria (PSB), Biofertilizer, salinity affected soil.

I. INTRODUCTION

Microorganisms are integral to the soil phosphorus (P) cycle and as such play an important role in mediating the availability of phosphorus to plants. The concept of microbial enhancement of phosphorus availability to plants is not new. (Gerretsen, 1948) showed that pure cultures of soil bacteria could increase the phosphorus nutrition of plants under controlled conditions through solubilization of precipitated forms of calcium (Ca) phosphates. Phosphorus (P) is one of the major essential macronutrients for plant and is applied to soil in the form of phosphatic fertilizers. In soil inorganic and organic forms of phosphorus is present. The inorganic forms of the element in soil are compound of calcium, iron, aluminum and fluorine. The organic forms are compounds of phytins, phospholipids and nucleic acid which come mainly by way of decaying undergrowth. Therefore, soils containing high organic matter are also rich in organic forms of phosphorus [1]. Soil microorganisms enhance plant nutrient acquisition. They are involved in a wide range of biological processes including the transformation of insoluble soil nutrients [2].

Phosphate solubilizing microorganisms (PSM) play a significant role in making phosphorus available to plants by bringing about favorable changes in soil reaction in the soil microenvironment leading to solubilization of inorganic phosphate sources. Some microorganisms associated with different plant rhizosphere are able to solubilise inorganic insoluble P salts. *Pseudomonas* and *Bacillus* are two important genera of soil bacteria with promising activity of phosphate solubilisation[3-4].

Many soil bacteria chiefly those belonging to the genera *Bacillus* and *Pseudomonas* posses the ability to bring insoluble phosphates in the soluble forms by secreting organic acids. These acids lower the pH and bring about the dissolution of bound forms of phosphorous. These bacteria are commonly known as phosphor-bacteria. They can be applied either through seed or soil application. Phosphorus, both native in soil and applied to inorganic fertilizers becomes mostly unavailable to crops because of its low level of mobility and solubility and its tendency to become fixed in soil. PSM increases the bioavailability of soil insoluble phosphorus for plant use [5].

II. MATERIALS AND METHOD

A. Sample Collection

The study total 128 soil samples were collected in sterilized container from Amravati district (Amravati tehsil-10, Daryapur tehsil - 43, Bhatkuli tehsil -31, Anjangaonsurji tehsil -23, Achalpur tehsil -03, and Chandurbazar tehsil -18). The soil suspension was prepared by mixing 1 g of soil sample in 9 mL distilled water then supernatant discarded and soil sample was point inoculated on previously prepared and sterilized pikovaskaya's agar plates. Then the pikovaskaya's agar plates were incubated at 28±2 oC for

24–48 h. And after completion of incubation time, Zone of phosphate solubilization was recorded. The colonies that showed clear zone of solubilization were further subculture on pikovskaya's agar plates.

B. Microscopic study of Bacteria and fungi

Microscopic study of the Size, shape, arrangement motility, grams staining was done for morphological study, the stained smear was observed under microscope (Oil immersion lance-100x). The fungal isolates were identified up to generic level based on their colony morphology and microscopic examination as outlined in the manual of [6]

C. Identification Of Bacterial Isolates Through Biochemical Test

The PSBs isolated from salt affected soils was identified up to generic level based on morphological, cultural, enzymatic test and biochemical tests as specified in Bergey's Manual of Determinative Bacteriology [7].

D. Phosphate Solubilization by Plate Assay

Solubilization of tricalcium phosphate was detected in Pikovskaya's Agar medium [8]. Each isolate was point inoculated at the center of Pikovskaya's Agar plate and incubated for 24 – 48 h. The developments of clear zone around the colony indicated phosphate solubilizing activity. The zone of solubilization was observed around the colony and diameter was measured.

E. Preparation Of Production Media Of Phosphate Solubilising Bacteria As Starter Cultures

After the screening of the PSB bacterial strains from the pure culture slants the bacterial strains were transferred to the liquid broth which was also the production media and as well as the starter culture for the growth of cells. Production media is that media in which the number of viable bacterial cells of that particular bacteria increases because that bacteria is grown in that particular media only. Thus, in phosphate solubilising bacteria strains were grown in Pikovasky as production media (Protocol followed for growth of PSB). Thus a 100ml of separate conical flasks were taken and PVK media was prepared after ph adjustments and autoclaved. Then inside the laminar airflow the pure cultures marked in the pure culture slants were transferred to the PVK production media conical flasks by the help of uncontaminated inoculating loop. Then the conical flasks were put in the rotary B.O.D shaker for 1 week or 7 days. The viable cell count in the production media or the liquid broth was found to come up to 109 CfU/ml. Then for the mass production of PSB bio-fertilizer the inoculums from these starter cultures were transferred to larger flasks.

F. Mass Production Of Psb Bio-Fertilizer And Preparation Of Inoculums

The Phosphate solubilising bacterial strains in the starter cultures were needed to be grown in large scale for which their mass production were required. So larger conical flasks of 1000 ml were taken and then again starter cultures were transferred to these larger conical flasks containing the appropriate growth media in aseptic conditions for small scale production and for large scale production again 1 liter of the starter cultures were put into the fermenter. Finally continuous agitation and proper aeration was complete for about 1 week. The flasks were checked for time to time for the growth of the cell mass and that they were free of any contamination. After 1 week the cell population increased up to 109 cells/ml or 109 cfu/ml load in the larger conical flasks. Then the conical flasks were stored in cool temperatures so that they can be mixed with proper carrier materials. Moreover it is not advisable to stay the conical flasks for long time in storage because of the loss of cell load.

G. Carrier Material Preparation

In this experiment for the inoculation to be made charcoal was used as carrier material. There are many steps for preparation of the carrier material. The steps are discussed below- First about collection of charcoal powdered. Then by the help of mortar and pestle the entire coal was crushed to dried crushed form. After crushing also the remaining pieces were further powdered by the help of mixer and grinder. The dust form of coal as charcoal was made and to it 1% calcium carbonate and wooden charcoal or activated charcoal was mixed and neutralized so that no contaminants were present.

H. Preparation Of Inoculum With Carrier Material (Mixing)

The mass produced bacterial cell cultures of PSB were taken out of storage and then the cell cultures were mixed with the sterilized carrier materials in individual beakers. The mixing of the carrier materials and the production media were in the ratio 2:1 where 1 part of production media was mixed with 2 parts of carrier material or in other words 30:60 ratios of both. It was done manually and

under aseptic conditions. The cell count of that carrier mixed culture was found to be 108 CFU/gm. The bio-fertilizers were filled in polythene bags which are advised to be of 250 gm. Then the packets were left in room temperature for curing.

I. Effect of this isolates on test plant

In present study used 24 pots, each pots containing 1 kg soil sample which were collected by salinity soil of study area. And each pot added soybean seed. Out of 24 pots, eight pots selected were for control and it was containing only soil and soybean seed. And other out of sixteen pot eight pot selected for checking effects of chemical fertilizer so it was contain 1 kg soil and soybean seed and add 1gm of chemical phosphate fertilizer. Other remaining eight pots were selected for check effects of PSB, so in these pots add 1kg soil, soybean seed and 1gm prepared biofertilizer. Then supply daily equal volume of water and observed growth up to 90 days from first date seedling.

III. RESULT AND DISCUSSION

The present section describes the results obtained during the study total 128 soil samples in Amravati district (Amravati tehsil-10, Daryapur tehsil -43, Bhatkuli tehsil -31, Anjangaonsurji tehsil -23, Achalpur tehsil -03, and Chandurbazar tehsil -18) Collected and analyzed, total 34 isolates were isolated from salinity affected soils Out of 34 isolates 21 PSB and 13 fungi were found, but only 3 fungi species showed significant zone of P solubilization. Among 21, only 8 PSB bacterial culture showed variation and others were repeated. A clear halo zone was formed around the colonies after 2 days of incubation on solidified Pikovaskaya's agar plates and all phosphate solubilizing bacteria and fungi were selected and sub cultured on Pikovaskaya's agar plates for further studies. In the Present study, total eight bacterial species and three fungi species were isolated and identifed on the basis of microscopic study, cultural study, Biochemical study and 16S RNA sequence. The isolated bacteria were named as Bacillus subtilis, Pseudomonas putida, Pseudomonas striata, Bacillus polymyxa, Bacillus megaterium, Bacillus cereus, Enterobacter cloacae, Micrococcus luteus, and Fungi- Trichoderma harzianum, Aspergillusniger, Penicilliumrugulosum. Characterisations were made following microbiology, a laboratory manual (Cappucino and Sherman, 1982).

Detail of biochemical and microscopic result given in following table no. (1).

Isolates	Gram	shape	motility	Indole	MR	VP	Citrate	Oxidase	Catalase	Nitrate	urea	starch	Gelatin	H ₂ S	D	Ga	Tr	Ma	Su	La	Fr	Ar	Ra
PSB 1	+	Ro d	+	-	-	+	-	-	+	+	+	+	+	-	+	-	-	+	+	-	-	-	-
PSB 2	-	Ro d	+	-	-	-	+	+	+	+	+	-	-	-	+	-	-	-	-	-	+	+	-
PSB 3	-	Ro d	+	-	-	-	+	+	+	+	+	-	-	-	+	-	-	-	+	-	+	+	-
PSB 4	+	Ro d	+	-	-	+	-	-	+	+	-	+	+	-	+	+	-	+	+	-	+	-	-
PSB 5	+	Ro d	+	-	-	+	+	-	+	+	-	+	+	+	+	+	-	+	+	+	+	-	-
PSB 6	+	Ro d	+	-	-	+	-	+	+	+	-	+	+	-	+	-	-	-	-	-	-	-	-
PSB 7	-	Ro d	+	-	-	+	+	-	+	+	+	-	-	-	+	-	+	+	+	-	-	+	+
PSB 8	+	Co cci	-	-	-	-	-	+	+	-	+	-	+	-	+	-	-	-	-	-	-	-	-

Table no. 1- Biochemical test of isolated strains

A. Fungal identification

Identification of fungal species was done as per the manuals of [9-10] After isolation of fungal isolate it was sub cultured on the PDA slants Phenotypic characteristics used as a means of identification for fungi rely on microscopic morphology for accurate and correct identification [11]. In this study, Trichoderma species cloud was growing at 28-35°C in 3-4 days. The strains belonging to

the genus *Aspergillusnigri* characteristically present dark-brown to black conidia, with uniseriate or biseriate conidiophores, spherical vesicles and hyaline or lightly pigmented hyphae near the apex [12]. In present study macroscopically, this fungus can be identified growing on substrates producing colonies of felt like yellow to white hyphae, turning black with the formation of conidia. Morphological identification of the potential *Trichoderma* isolates was performed using an online interactive key [13] based on the colony appearance and pigmentation, the presence or absence of sweet coconut smell, growth rate at 35°C, the presence or absence of pustules on CMD, the sizes of conidia, the branching patterns of conidiophores, and the presence or absence of chlamydospores.

Detail of isolated fungi cultural character given in following table

Fungi	Colony diameter	Colony color	Conidia	Mycelium	Exudates	Colony edge	Reverse
Fungi- 1	80-90mm	Yellow green	Green	Watery white	yellowish watery droplets	smooth	Colorless
Fungi-2	90 mm	Dark black	Black	Dull white	Nil	smooth	Florescent yellow and wrinkled mycelia
Fungi-3	30–45 mm	Yellow green	Grey	White	Clear rosy	smooth	colorless

Table no. 2- Cultural characters of fungi

B. Genotypic Identification

All the 11 isolates were deposited in gene bank at GSBTM with particular accession number (BAB) shown following table. 16S rRNA gene sequences of the strains obtained after sequencing were identified using BLAST at the National Centre for Biotechnology Information web site: www.ncbi.nlm.nih.gov and submitted to GenBank EMBL with accession number assigned by them. After 16S rRNA sequence analysis, identity of 11 isolates. Most of the isolates showed 98 to 100% of similarity with sequences in the EMBL data. [14] Suggested that a 16S rRNA gene sequence similarity of 97 % should become the boundary for delineation of prokaryotic species, which has been well accepted among microbiologists.

C. Phylogenetic Tree

Molecular analysis by the 16S rDNA identification technique was adopted in this study. These excellent markers for the clarification of bacterial phylogeny are ribosomal ribonucleic acids. In this study, we used gene sequences from to determine the phylogenetic relationships among the tested isolates. The neighbor-joining tree was subjected to the numerical re-sampling by bootstrapping, and the resulting bootstrap values were observed at the tree branch nodes. The identification of phylogenetic neighbours was initially carried out by the BLAST [15] and MEGA BLAST [16] programs against the database of type strains with validly published prokaryotic names [17].

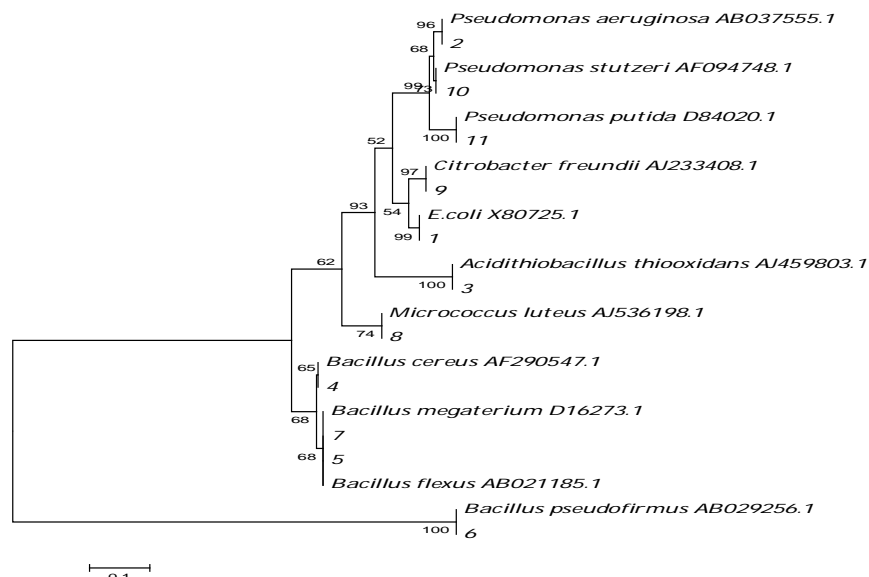


Fig no. 1- Phylogenetic tree of isolated PSB

D. Determination of Efficiency of Phosphate solubilization

A clear halo zone was formed around the colonies after 2-4 days of incubation on solidified Pikovaskaya's agar plates and all phosphate solubilizing bacteria and fungi were selected and sub cultured on Pikovaskaya's agar plates for further studies.. That is Bacillus cereus, Bacillus megaterium, Bacillus subtilis, Bacillus polymyxa, pseudomonas straita, pseudomonas putida., Micrococcus lotous., Enterobacter cloacae., fungi (Aspergillus niger, Penicillium rugulosum. and trichoderma harzanium) Out of this isolate fungi (Aspergillus niger, Penicillium rugulosum. and trichoderma harzanium)) having efficiency of Phosphate solubilization was more as compare to other isolated phosphate solubilizing bacteria that was (284, 220, 276) But Enterobacter cloacae having efficiency of Phosphate solubilization was less as compare to other isolated phosphate solubilizing bacteria that was (127). Efficiency of Phosphate solubilization was determined by plate assay using Pikovaskaya's Agar Medium.

Phosphate solubilisation efficiency was assayed visually and the solubilisation index (SI) for each isolate was calculated as the ratio of total diameter of phosphate solubilization to colony diameter [18].

Detail of zone of efficiency given by in following table no 3.

% of Efficiency of PSB was calculated by using following formula

$$\text{Efficiency of phosphate solubilization} = \frac{\text{Solubilization diameter}}{\text{Diameter of colony}} \times 100$$

Sr. No.	PSB and Fungi strain	Colony Diameter	Solubilization Diameter	% Efficiency 48-72 Hr
1.	Bacillus subtilis	0.9	1.4	155
2.	Pseudomonas putida	0.5	1.2	240
3.	Pseudomonas straita	0.6	1.4	233
4.	Bacillus polymyxa	0.8	1.1	137
5.	Bacillus megaterium	1.2	1.8	150
6.	Bacillus cereus	1.2	1.6	133
7.	Enterobacter cloacae	1.1	1.4	127
8.	Micrococcus lotus	1.3	3.6	276
9.	Trichoderma harzanium	1.3	3.6	276
10.	Aspergillus niger	1.3	3.7	284
11.	Penicillium rugulosum	0.5	1.1	220

Table no 3 - % of efficiency

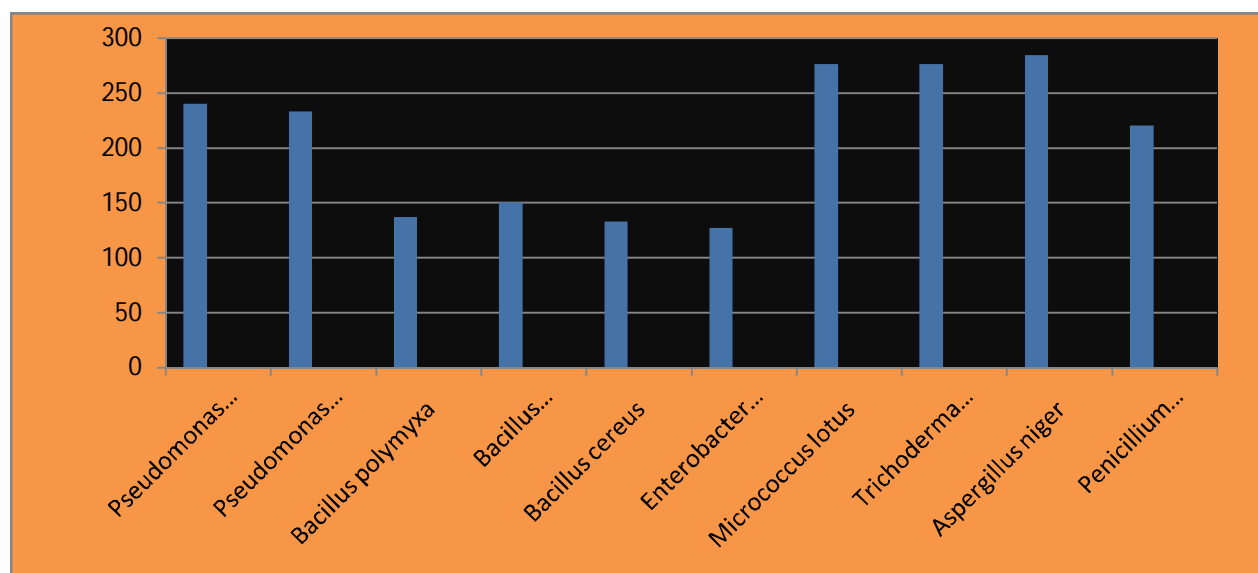


Fig No 2- Determine % of Efficiency

E. Pot experiment of Soybean plant

Following table shows the growth promotion in *Soybean* test plants as compared with control after 90 days.

Sr. No.	Plant of Soybean	Shoot length (cm)	Root length (cm)	Leaf width	No. of leaf	No. of branches
1.	A1-Control (seeds)	66	8.9	3.2	32	10
2.	A2 (seeds + Phosphate fertilizer)	68	9.4	3.2	36	12
3.	A3 (seeds + Isolated culture)	74	9.9	3.3	48	15

Table no. 4- Comparison of average growth test plant with control (24 pots)

In the present investigation effect of combination of biofertilizer and soil were studied on the growth parameters of soybean plant. The inoculation of isolated PSB had a more stimulating effect on the assimilation and solubilization of phosphate than uninoculated control. The lowest values of soybean growth parameters were obtained by soil without inoculation (Control). It was obvious that addition of biofertilizer inoculated phosphate solubilising microorganisms caused an enhancement in plant growth parameters. This study confirms that phosphate solubilizing microbial inoculants improved the estimated characters compared with untreated control. Many phosphate solubilizing bacteria are reported as plant growth promoter [19-20]. It is well established that introduction of plant growth promoting bacteria.[21] Reported that PSM increased phosphorus accumulation in plants, yield of pea and barley.

IV. CONCLUSION

It is concluded from the present study A total of 11 strains (*Bacillus cereus*, *Bacillus megaterium*, *Bacillus subtilis*, *Bacillus polymyxa*, *pseudomonas putida*, *pseudomonas straita*, *Micrococcus lotus* , *Enterobacter cloacae*, fungi -*Aspergillus niger*, *Penicillium rugulosum* and *Trichoderma harzanium*) were isolated from salinity affected soil in Amravati district are very useful for increasing solubilization of inorganic insoluble phosphate. This isolates are very important for increasing crop yield which is taken in salinity affected soil in productivity of soybean (*Glycine Max*) plant etc. Salinity is a serious environmental issue, as it limits crop growth and drastically reduces productivity. Therefore, in addition to these isolate increase crop productivity because these isolate not only solubilize phosphorous but also increases nitrogen uptake. In this study, the Cheapest and easily available carrier (charcoal powder) were utilized for the production of nutrient rich phosphate solubilizing biofertilizer for salinity affected soils better growth of plant. From the study it was observed that the fungi (*Aspergillus niger*, *Penicillium rugulosum* and *Trichoderma harzanium*) have the more solubilizing ability of inorganic insoluble phosphate than bacteria, i.e., *Bacillus cereus*, *Bacillus megaterium*, *Bacillus subtilis*, *Bacillus polymyxa*, *pseudomonas putida*, *pseudomonas straita*, *Micrococcus lotus* , *Enterobacter cloacae*., Hence the application of biofertilizer prepared by above mentioned fungi should be helpful to increase the crop yield in salinity affected soil by solubilizing large concentration of inorganic insoluble phosphates

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