Isolation and Characterization of Phosphate Solubilising Bacteria from Rhizospheric Soil Samples

Omkar Shankarrao
School of Life Sciences, Swami Ramanand Teerth Marathwada University, Nanded.431606, Maharashtra, India.

Abstract

Most of the soil phosphate found in insoluble form making unavailable to plant. To circumvent the phosphate deficiency, phosphate solubilizing microorganisms play an important role in supplying P to plants. In the present study total 28 Phosphate Solubilising Bacteria were isolated from rhizospheric soil of Neem, Mango and Jatropha plant. The solubilization index of each isolates was determined on Pikovskaya agar medium. The isolate M (III), M (III) col-2, M (III) col-4, N (b) col-1, N (c) col-2, J(A) and J-C col-2 showed high P solubilization potential having SI =2.11 - 3.35 recorded and quantitatively solubilized 160, 182, 270, 164, 200, 228 and 182 mg/ml P respectively after 7 day incubation .The isolates were identified and characterized for the plant growth promoting activities such as production of Ammonia, Indole Acetic Acid, Cell wall degrading enzyme; cellulase, chitinase and proteolytic enzyme and antagonistic ; against plant pathogenic fungi and bacteria. The P solubilization was accompanied by reduction in pH of the medium. The result indicated the rhizospheric soil is the rich source for isolation of phosphate solubilizing bacteria which promote the plant growth by more than one PGPR trait and have wide application in soil ecology.

KEYWORDS: Phosphorus, Plant growth promotion activity, Phosphate solubilising bacteria.

Introduction:

Phosphorus (P), next to nitrogen is the second important micronutrient required for plants growth and development. This nutrient is acquired by plants from the soil solution mainly in the phosphate anions form as $H_2PO_4^-$ and HPO_4^{2-}. To obtain maximum yield of crops chemical fertilizers are regularly applied in the field. But more than 70-90% of the applied phosphate fertilizers get fixed in soil with cations (Ca^{2+}, Mg^{2+} ,Fe^{3+} and Al^{3+})or adsorbed to Fe-oxides and Al-oxides, Al-silicates, and Ca-carbonates, depending on the soil properties and making unavailable for plant uptake thus increasing the phosphorus requirement of the plant(Ahmad Ali Khan,*et.al.*,2009).

Microorganisms found in soil such as bacteria, fungi and actinomycetes have fundamental role in the biogeochemical cycling of P in natural and agricultural soil ecosystems. However microorganisms in the rhizosphere are found to be more in population and having high metabolic rate rather than in non rhizosphere soil (Tamilarasi *et.al.*,2008). Among rhizospheric microorganism, bacteria having largest population termed as Rhizobacteria of which only 2.5% exert beneficial effects on plant growth and development are called as Plant Growth Promoting Rhizobacteria (PGPR).The

PGPR exhibit various activities such as phytohormone production, suppressing the growth of deleterious microbes, nitrogen fixation, solubilisation of phosphate and other nutrients which play an important role in plant development(Hilda ,*et.al.*,2000). .

Microorganisms involved in phosphorus acquisition include bacteria, actinomycetes (Banik S.*et.al.*, 1982) & fungi. The soil fungi, particularly those belonging to the genera *Penicillium* , *Aspergillus, Rhizopus* and *Fusarium* have been reported to solubilize insoluble phosphates by secreting weak organic acids (Maliha *et al.*, 2004 and Chunqiao. *et al.,2009).* Among the soil bacterial communities, strains from *Pseudomonas* , *Bacillus* , *Enterobacter* and endosymbiotic *Rhizobium* have been described as effective phosphate solubilizers. *Bacillus megaterium, B. circulans, B. subtilis, B. polymyxa, B. sircalmous, Pseudomonas striata,* and *Enterobacter* could be referred as the most important strains (Jose *et.al.*, 2005) . Bacteria are more effective in phosphorus solubilization than fungi (Alam *et al.*, 2002).The bacterial P solubilization activity due to secretion of organic acids. The organic acids oxalic, citric, formic, acetic, propionic, lactic, succinic and gluconic acid which chelate the cation bound to phosphate and being converted to soluble forms through their hydroxyl and carboxyl groups and production of acid /alkaline phosphatase enzyme (Chen *et.al.*,2006).

The aim of present study was to isolate the efficient PSB from rhizospheric soil samples and characterize them for plant growth promoting activities.

MATERIALS AND METHODS

Sample collection and isolation phosphate solubilising bacteria (PSB)

A total nine of rhizospheric soil samples were collected from campus area of S.R.T.M. University, Nanded, (Maharashtra). From nine samples, three rhizospheric samples were collected from jatropha plant, three from neem and remaining from mango rhizospheric soil. Soil samples were collected at a depth of 10 cm, in sterile polythene bags and stored in refrigerator for actual experimental work.

10 gm of soil sample was placed in 100 ml distilled water, after shaking for 30 min. 1 ml of sample was serially diluted up to 10^{-6} in 10 ml sterile saline solution. 0.1 ml of each appropriate diluted soil samples were spread on Pikovskaya's agar (PKA) plates (Pikovskaya, 1948) and incubated it for 4 days until highest number of colonies developed. Amphotericin B 200 mg/L added in PKA to avoid fungal growth.

Solubilisation index (SI):

Thick bacterial suspension spot of each isolates were inoculated on Pikovskaya's agar plates and incubated for 7 days at 37 ^{0}C and SI were measured daily from day 2to 7 by using following formula (Edi-Prempno et.al. 1996).

$$SI = \frac{\text{(Colony diameter + halozone diameter)}}{\text{Colony diameter}}$$

Quantitative estimation of solubilized phosphates & pH determination:
Each isolate was inoculated in 30 ml PVK broth of 150 ml conical flask and incubated at 30°C & 150 rpm for 5 days. Broth culture was centrifuged at 10,000 rpm for 20 min and the cell free supernatant (CFS) was used to record the change in pH and solubilized P in the culture medium. The pH of the culture filtrate was measured with pH meter and mobilized P was estimated by phosphomolybdic blue colour method (Jackson, 1973).

Antagonistic assay:

The bacterial isolates were grown in 30 ml Nutrient Broth of 150 ml conical flask for five days at 30^{o} c and 150-rpm on shaking incubator. Cultures broth medium were centrifuged at 10000 rpm for 20 min and cell free supernatant were used for Antagonistic assay by well agar assay.

Suspension of Six fungal pathogen such as *Alternaria brassicicola* NCIM 1045 , *Alterrnaria solani* ATCC 11785, *Rhizopus oryzae* NRR L2582, *Aspergillus niger* MTCC 1781, *Helminthosporium gramineum* NCIM 1070, *Ustilago maydis* PRL1549 and *Sclerotium rolfsii* NCIM 1084 were spread on Potato dextrose agar (PDA) plate and five bacterial pathogens such as *Escheritia coli* ATCC 33684, *Staphylococcus aureous* ATCC 9144, *Salmonella typhimurium* ATCC 23564, *Pseudomonas aeruginosa* NCIM 8295, *Klebsiella aerogene* ATCC 8329 were spread on nutrient agar plates. Then 10 mm well was made with the help of sterile cork borer and 200 µl CFS was pipette into the well and sterile with uninoculated medium was used as control. The plates were incubated at 30^{0}C and the zone of inhibition was measured around the well after 24-48 hrs for antibacterial assay and 48 to 72 hrs for antifungal assay determinations.

Indole production assay:
Each isolate were inoculated into 1% tryptone broth (5ml/tube) and incubated at 30^{o}C , for 48 hrs .Then 1ml of Kovac's reagent was added , the tubes were gently shaken and allowed the tubes to stand in order to permit the reagent to come on the top .The tubes were examined for development of colour. Uninoculated tube with 1% tryptone broth was used as control.

Assay for NH_3 production:
Production of ammonia was determined in peptone water (Chaiharn *et.al.*, 2008). Freshly grown cultures were inoculated into 10 ml peptone water and incubate for 48 hrs at 30^{o}C. Nessler's reagent (0.5ml) was added to each tube and observed for ammonia production. Uninoculated sterile medium was used as control.

Determination of cell wall degrading enzyme assay:
Protease assay:
The protease activity determined using skim milk agar medium, which contained. 5 gm pancreatic digest of casein, 2.5 gm yeast extract, 1 g glucose, 7% skim milk solution and 15 g of agar. Bacterial cells were spot inoculated and after 5 days incubation at 37°C observed plates zone around colony.
Chitinase assay:

The chitinase activity tested on chitin agar containing 1.62 gm nutrient broth, 0.5 g NaCl, 6 g M9 salts, 8 g colloidal chitin and 15 gm agar/liter distilled water. Spot inoculated of isolates observe zone of clearance around the colony after 5 days incubation at 30°C.

Cellulase assay:

The cellulase production assay done on nutrient agar plate containing 10 g of cellulose by spot inoculation. After 5 days of incubation at 28°C, colonies surrounded by clear halos were considered positive for cellulase production.

Results and discussions:

The total nine rhizospheric soil samples of Neem(N), Jatropha(J) and Mango(M) plants were collected with varying properties such as for site J-B, J-C,N-C,M-I,&M-II were having white, rocky, uncultivated soil & for site J-A, N-a, N-b, &M- III having black cottony, cultivated with good fertile soil. The occurrence of P solubilizing bacteria in rhizospheric soils was very well documented. In the present study 28 bacterial isolates were isolated on Pikovskaya's agar (PKA) plates .Generally bacterial isolates with clear halozone around the colony were selected. (Fig.1)

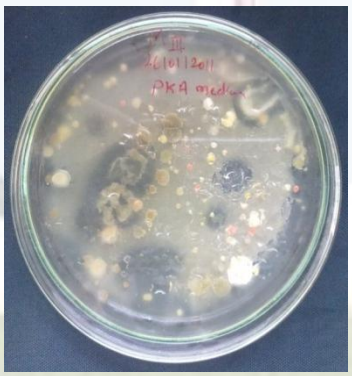


Fig: 1.Isolation of PSB on PVK agar plates.

Solubilisation index based on colony diameter and halozone formation for each isolates were determined on PKA agar plates .Generally it was observed that, as colony diameter increases zone of clearance around colony also increases by solubilising tricalcium phosphate in the medium. Among all isolate, 8 potent P solubilizer such as J-A, J-C (col 2), M-III (col 2) ,M-III (col 4) ,M-III , M-III (2),N-c (col 2) and N-b(col 2) were selected for further study which showed consistence SI in the range 2.11 to 3.38. (Table.1 & Fig.2).Although Fluctuations in SI were observed during the seven days observation period. In most of the cases it gradually increased while in few cases such as M-II (2), M-III (col2), J-A it first decreased and then and then increased (table 1).

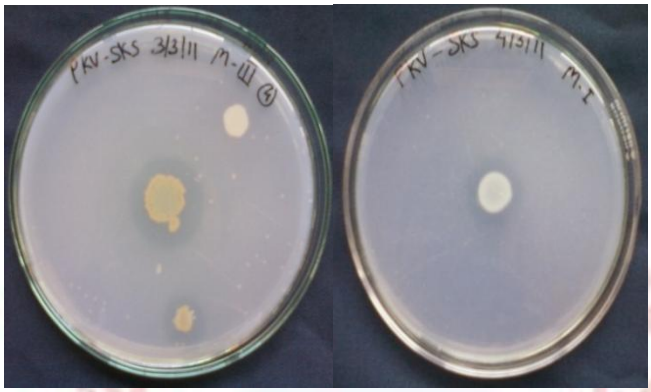


Fig 2: showing Solublization Index (SI) of isolate M-III (4), and M-I

Most of the bacterial isolates lost their ability to solubilize P on Pikovskaya's agar medium during repeated sub culturing but only 8 isolates such as J-A, J-C (col 2), M-III (col 2) ,M-III (col 4) ,M-III , M-III (2),N-c (col 2) and N-b(col 2) showed persistence to this activity .

Table 1: Showing Solubilization index of PSB isolates.

Sr.no.	isolates	Solubilization Index through seven days in (cm)						
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
1.	N-(b) col- 2	1	1	1	2.19	2.25	2.15	2.22
2.	N-c Col-2	2.11	2.52	2.52	2.69	2.69	2.52	2.52
3.	M-III	2.7	2.5	2.8	3.00	3.07	3.15	3.23
4.	M-III (2)	1	1	1.52	2.52	2.84	2.69	2.69
5.	M-III Col- (4)	1	2.20	2.37	2.56	2.75	3.15	3.40
6.	M-III Col- (2)	2.52	2.84	2.69	2.69	3.00	3.07	3.15
7.	J-A col(1)	1	1	3	3.15	3.00	2.94	2.81
8.	J- C Col 2	2.3	2.4	2.5	2.1	2.0	2.2	2.2

These isolates were identified on the basis of colony characteristics, microscopic observation and biochemical test. The isolates such as M-III (col 4), M-III and N-c (col 2) were gram positive ,rod shaped ,endospore forming ,catalase positive and nitrate reduced to nitrite .Therefore these isolates are among the genus Bacillus. Remaining were gram negative rods J-A and N-b (col 2) and cocci J-A, M-III (2) which may belongs to genus *Psuedomona , Rhizobium , Azotobacter or Enterobacter.*

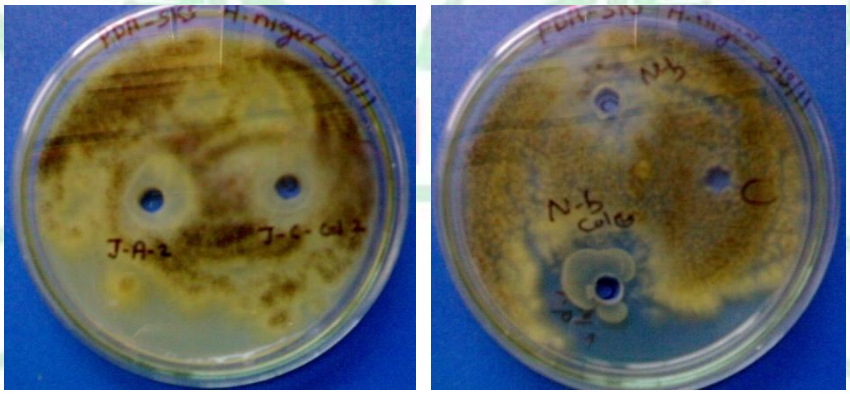
The soluble phosphorus estimated in the PVK broth medium in the range between 140 to 270 µgm/L (Table 2). Xuan Yu *et.al.*, 2011 also reported the 34 bacterial isolates which solubilize the P in the range 81-233 µgm/L with associated decrease in pH of the broth medium. Maximum phosphorus solubilisation was recorded by isolates M-III (col 4) (270 µgm/L) followed by N-c (col 2), J-A, & J-C (col 2) M-III (col 2), N-b (col 2) &J-A (col 1) with drop in pH of the medium. Mittal *et al.*, (2008) also suggested that values

of P solubilizing activity may be correlated with levels of acid production. Pratibha Vyas *et.al.,* 2006 also reported maximum drop in pH was associated with higher level of phosphorus solublization. Thus in the present study also statistically inverse relation between pH and soluble phosphate concentration was observed.

The antagonistic activity of 8 PSB isolates were determined against six phytopathogenic fungi and five bacteria by agar well diffusion assay (Fig 3).Isolates N-B (col1),N-c(col-2),J-C(col 2) ,M-III and M-III (col-2) showed potent antifungal activity against *H. gramineum* and *R. oryzae* (Fig.3B) .Isolates N-B (col-1), M-III and M-III col-2 showed antagonistic activityagainst A.niger and U.maydis (Fig.3A). Isolates N-B (col -1), M-III and M-III (col-2) showed good antifungal activity against *R. oryzae, H. gramineum, A. niger* and *U. maydis*. Comparitively, *R. oryzae, H. gramineum, A. niger* and *U. maydis* showed more sensitiveness to tested isolates than *A. brassicicola* , *A. solani* and *S. rolfsiiI*.

The antifungal activity of bacterial isolates may be due to secretion of cell wall degrading enzyme or antibiotics in to the environment. Salem *et.al.*, 2012 reported the antifungal activity of bacillus subtilis due to secretion of several enzyme and Leon *et.al.*, 2009 report the antagonistic activity of indigenous and bacillus from Soybean rhizosphere due to secretion of antifungal compound. Bacilli are well reported for production for peptide and non peptide metabolite having potential antifungal activity (Ahlem *et.al.*, 2010).

All isolates except J-C (col 2) have antibacterial activity against *S.aureus* .Among five bacterial pathogen tested *S.aureus* showed more sensitiveness, followed by *Pseudomonas aeruginosa* and *Salmonella typhimurium.*



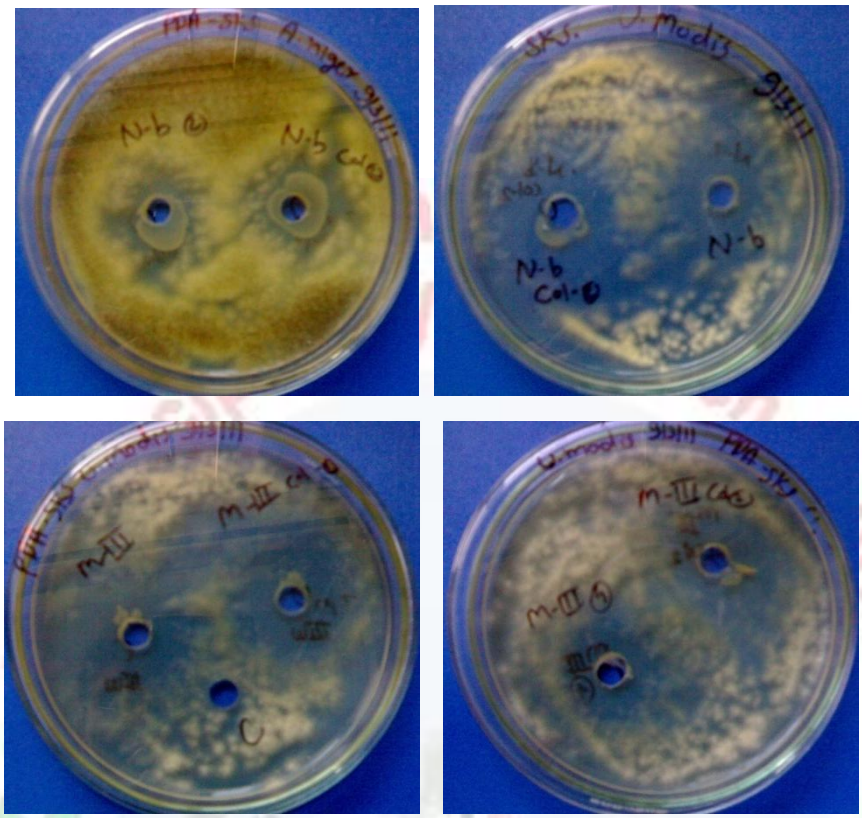


Fig.3.A: Antifungal activity of PSB isolates (N-B (col-1), M-III and M-III col-2) against *Aspergillus niger* and *Ustilago maydis*.

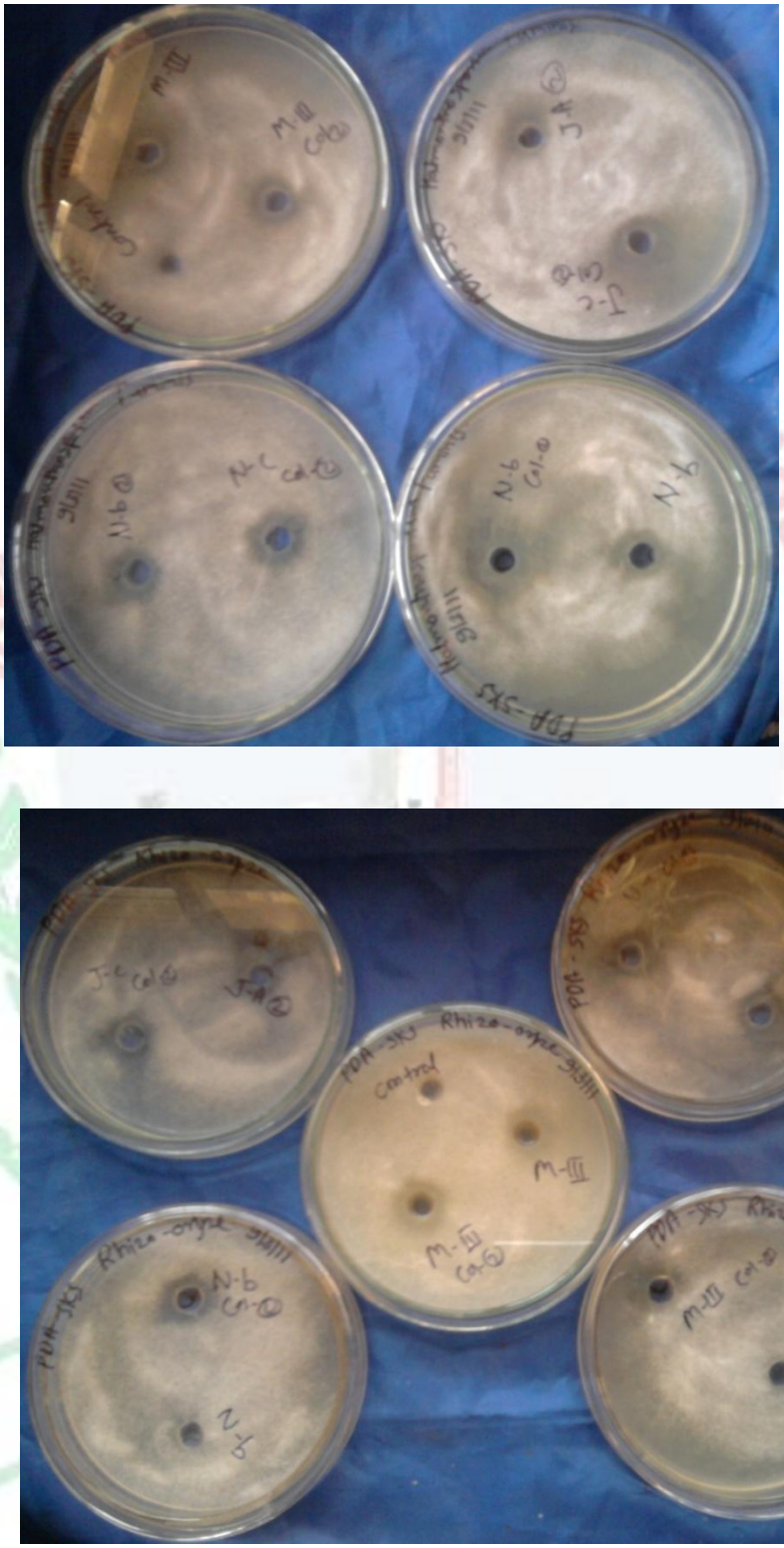


Fig. 3.B: Antifungal activity of PSB isolates (N-B (col1), N-c (col-2), J-C(col 2) ,M-III and M-III (col-2))against *H. gramineum and R. oryzae*

Development of a cherry (deep) red color in the top layer of the tube is a positive for Indole production. All isolates showed positive test for Indole production (table 2), whereas control did not show. Complex interaction between plant and bacterial producing indole might be because IAA secreted by a bacterium may promote growth directly by stimulating plant cell elongation or cell division.

Table 2: Showing plant growth promotion activity, pH change and estimated phosphate from culture broth of PSB isolates.

Sr.No.	isolates	Indole production	Cell wall degrading enzymes			NH_3 production	pH	Estimated available phosphate (µgm/L).
			Protease	Kerotinase	cellulase			
	Control	- Ve	- Ve	- Ve	- Ve	- Ve	6.5	82
1.	M (III)	+ Ve	+ Ve	- Ve	+ Ve	+ Ve	6.0	160
2.	M(III) col 2	+ Ve	+ Ve	+ Ve	- Ve	+ Ve	5.1	182
3.	M (III) 2	+ Ve	+ Ve	- Ve	+ Ve	- Ve	5.5	140
4.	M(III) Col 4	+ Ve	- Ve	- Ve	+ Ve	- Ve	4.2	270
5.	N(b) Col 2	+ Ve	- Ve	- Ve	+ Ve	+ Ve	5.8	164
6.	N(c) col 2	+ Ve	- Ve	- Ve	- Ve	+ Ve	4.3	200
7.	J(A)	+ Ve	- Ve	- Ve	+ Ve	- Ve	4.9	228
8.	J- C Col 2	+ Ve	+ Ve	+ Ve	+ Ve	+ Ve	6.1	182

(+ = Positive, – = Negative)

Production of ammonia was a common trait in all phosphate solubilising bacteria tested except M-III (2) & M-III (col 4) (table 2). In ammonification process bacteria convert the organic nitrogen, back in to ammonia (NH3). Development of brown to yellow color indicated a positive test for ammonia production.

From the present study, we demonstrate that the natural Neem, Jatropha and Mango rhizosphere soil can be the rich source for isolation of phosphate solubilizing microorganism. It may be due to high P requirements to Neem tree (*Azadirachta indica*) and other meditional plant (Phavaphutanon *et al* .,1996), or due to long term association and interaction between Neem root and microorganism found in the rhizosphere environments(Barriuso *et al*., 2005 and Lucas Garcia *et.al* ., 2001). Isolates such as M-III, M-III col-2 and N-b col 1 showed more than one PGPR trait such as phosphate solubilisation, antifungal & antibacterial activity and Phytohormone production. These isolates may promote plant growth directly, indirectly or synergistically in the soil environment. Therefore further need for field trial study which can be serve as efficient biofertilizer and biocontrol candidates for improving the plants growth.

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