Isolation and Characterization of *Ralstonia pickettii* - A Novel Phosphate Solubilizing Bacterium from Pomegranate Rhizosphere from Western India

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***Abstract*—In the field of agricultural microbiology attempts are being made to find novel microbes for use as efficient biofertilizers including phosphate solubilizing microbes (PSM). Particularly, research on use of microbes for improving availability of phosphorus continues to be a major area of focus. Here we report isolation of *Ralstonia pickettii* - a novel phosphate solubilizing bacterium from a unique source - rhizosphere soil of pomegranate as well as some other relatively less explored sources from different parts of Western India. 23/134 cultures were found to be potential phosphate solubilizing bacteria. Further, 8/23 isolates were tested for an array of plant growth promoting attributes *viz*. solubilization of inorganic phosphate, production of phosphatase enzymes, indole acetic acid, siderophores, etc. The three cultures that showed best phosphate solubilization under shake flask conditions were culture no. GN/PSB/PC2, identified as *Ralstonia* (574 ppm), culture no. GN/PSB/KC5 - *Burkholderia tropica* (400 ppm) and GN/PSB/CC2 - *Burkholderia cepacia* (~375 ppm). Some of the cultures were found to produce appreciable amounts of plant growth promoters like indole acetic acid and siderophores.**

***Index Terms*—*Ralstonia pickettii*, *Burkholderia spp.*, pomegranate, phosphate solubilizing bacteria, phosphatase, biofertilizers.**

I. Introduction

Plant growth requires the availability of multiple mineral and non-mineral nutrients in varying quantities. Precisely, sixteen chemical elements are known to be important to a plant's growth and survival. These elements form the various organic and inorganic nutrients required for plant development (Rodriguez and Fraga, 2013).

Phosphorus (P) is second only to nitrogen amongst the mineral nutrients most commonly limiting the growth of crops. Phosphorus participates in many of the reactions that keep plants and animals alive, and is thus essential for all living organisms (www.nzic.org.nz/ChemProcesses/soils/2D.pdf).

Availability of phosphorus is highly essential for plant growth although on an average it makes up only about 0.2% dry weight of plants [Keneni *et al*, 2010]. It forms a component of key biomolecules, for e.g. nucleic acids, certain proteins, coenzymes, and lipids. P is also involved in controlling key enzyme reactions and in the regulation of metabolic pathways and it also plays an important role in the formation of high energy bonds of ATP, the energy currency of the cell (Theodorou, 1993).

The ideal pH range of soil for optimum plant growth is 6.0-6.5. However, soil pH is rarely in this range and consequently several measures need to be taken to ensure nutrient availability to the plant [Guodong, 2014]. Phosphorous is found in two different forms in soil: inorganic and organic. The main inorganic forms of phosphorus in soil are H_2PO_4 - and HPO_4^{2-}. This is the form in which phosphorus is used by plants. However, these ions can also absorb/adsorb on the surface of other non-living solid matter in the soil. Such adsorbed phosphorus is then unavailable to plants; this means that phosphorus though naturally present in the soil, enough is not usually available for plants to grow well. Phosphorus levels are further reduced by grazing animals; and also it may be washed away by excess rain. To compensate for such losses, excess of chemical phosphate fertilizers are widely used.

Phosphorus as a plant nutrient can also have several impacts on the environment. Drastically high or low levels of phosphorus can have a severe and widespread negative impact on environmental quality. Excess P (including both soluble and insoluble forms of P) levels in the soil are usually a result of continuous and unscrupulous use of chemical fertilizers. High soil P levels lead to accelerated eutrophication of water bodies due to run offs. The principal environmen4tal problem

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related to low soil phosphorus is land degradation caused by agricultural depletion of soil nutrients making fertilizer application imperative. Application of phosphorus as fertilizers to soil is a necessity and cannot be avoided as most soils nowadays, are deficient in plant available phosphorus.

Soil phosphorus dynamics is characterized by physicochemical (sorption-desorption) and biological (immobilization-mineralization) processes. Large amount of phosphorus applied as fertilizer enters in to the immobile pools through precipitation reactions with highly reactive Al^{3+} and Fe^{3+} in acidic, and Ca^{2+} in calcareous or normal soils [Khan *et al*, 2009]. As a result, it is estimated that adequate P fertilization requires large amounts of P because up to more than 80% of the P fertilizer may be strongly absorbed or precipitated by the soil and not be immediately available to the crop (Hedley *et al*, 1995; *Phosphorus Fertility Management In Agro ecosystems*).

It is well known that a considerable number of bacterial species, mostly those associated with the plant rhizosphere, are able to exert a beneficial effect upon plant growth. Therefore, their use as biofertilizers or control agents for agriculture improvement has been a focus of numerous researchers for a number of years. This group of bacteria has been termed *plant growth promoting rhizobacteria* (PGPR). The mechanisms by which PGPR can exert a positive effect on plant growth can be of two types: direct and indirect. Indirect growth promotion is the decrease or prevention of deleterious effect of pathogenic microorganisms, mostly due to the synthesis of antibiotics. Direct growth promotion can be through the synthesis of phytohormones, N_2 fixation, reduction of membrane potential of the roots, synthesis of some enzymes [such as 1-aminocyclopropane-1-carboxylate (ACC)-deaminase] that modulate the level of plant hormones, as well as the solubilization of inorganic phosphate and mineralization of organic phosphate, which makes phosphorous available to the plants (Rodriguez *et al*, 1999).

Several scientists have examined the ability of different bacterial species to solubilize insoluble inorganic phosphate compounds, such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite, and rock phosphate (Rodriguez *et al*, 1999). It is generally accepted that the major mechanism of mineral phosphate solubilization is the action of organic acids synthesized by soil microorganisms. Consequently, P_i may be released from a mineral phosphate by proton substitution for Ca^{2+}. The production of organic acids by phosphate solubilizing bacteria has been well documented. Organic phosphate solubilization is also called mineralization of organic phosphorus, and it occurs in soil at the expense of plant and animal remains, which contain a large amount of organic phosphorus compounds. The mineralization of these compounds is carried out by means of the action of several phosphatases (also called phosphohydrolases). These dephosphorylating reactions involve the hydrolysis of phosphoester or phosphoanhydride bonds (Rodriguez, 1999).

II. Materials and Methods

All chemicals and reagents used were procured from Merck India Ltd. or from SRL Chemicals India. Certain microbiological media components were procured from HiMedia Labs, India.

All assays reported here were performed in triplicates.

A. Collection of Soil Samples

Soil samples were collected from the rhizosphere regions of pomegranate plants and other plants from different regions in western India (Table I). Samples were collected by carefully removing soil, ~2-3 cm from the surface around the root region of the plant and were taken into pre-sterilized glass bottles. The samples were stored at 4 ℃ till use. Normally samples were processed within two days after collection.

TABLE I: SOURCES FOR ISOLATION OF PSB

Rhizosphere Soil Sources	**Non-Soil Sources**
Cashew nut	Raw areca nut
Coconut	Raw coconut fruit
Garlic	Spoilt garlic bulb
Grapes	*Pandanus fascicularis* flower
Jack fruit	
Mango	
Pomegranate	
Rice	
Tomato	

B. Screening and Isolation of Phosphate Solubilizing Bacteria

Soil samples were screened for presence of phosphate solubilizing bacteria using the dilution plating technique on Pikovskaya's agar containing (in g/L): Glucose: 10.0, $(NH_4)_2SO_4$: 0.5, KCl: 0.2, $MgSO_4.7H_2O$: 0.1, $Ca_3(PO_4)_2$: 5.0, Yeast Extract (HiMedia): 0.5, trace amounts of $MnSO_4$ and $FeSO_4$ and 1.5% agar (HiMedia). The medium was sterilized by autoclaving at 121 ℃ for 15 mins and plates were prepared. The autoclaved molten agar was swirled regularly to achieve uniform mixing of $Ca_3(PO_4)_2$. Samples were suspended in saline (10g in 100 cm^3) and placed on rotary shaker for about 3h. Suspension was serially dilutied 10-fold as per procedure described (University of Toledo, *Serial Dilution and Spread Plate Technique*, 2004). Samples were surface spread on Pikovskaya's agar and incubated at 28 ℃ until colonies formed. Colonies were observed for clear zones around them presumably due to solubilization of tri-calcium phosphate. Select colonies were purified, isolated and maintained as glycerol stocks using 40% glycerol *(*Stockinger Labs, *Glycerol Stocks*, 2001).

C. Determination of Solubilization Indices of Potential PSB

Solubilization index (SI) is the ratio of diameter of the zone of clearance compared to the diameter of colony.

$$SI = \frac{[diameter\ of\ clearance\ zone\ (including\ colony\ diameter\ in\ mm)]}{colony\ diameter\ (in\ mm)}$$

The solubilization index is a preliminary measure for identifying ability to solubilize tricalcium phosphate or other sources of insoluble phosphorus (Collavino *et al*, 2010).

D. Determination of Phosphorus Solubilizing Efficiency of Isolates

The procedure for determining amount of soluble phosphorus was adapted from the method described by Gaur A.C. (1990): Culture suspension - 0.5 cm^3 (OD_{540nm} adjusted to approx. 10^8 CFU/cm^3) was added to 50 cm^3 sterile Pikovskaya's broth (containing 1000 ppm P) in a 250 cm^3 Erlenemeyer flask and incubated at 28 ℃ for 120h at 180rpm. Samples were withdrawn at 24h intervals and centrifuged at 12000g for 10 minutes. 0.5 cm^3 of the supernatant was added to a 50 cm^3 volumetric flask to which 10 cm^3 of chloromolybdic acid was added. Volume was made up to ~40 cm^3 using distilled water; 2.0 cm^3 of chlorostannous acid was added and the volume was quickly made up to the mark using deionized water. The intensity of blue colour developed was measured in a spectrophotometer at 700 nm (Shimadzu Spectrophotometer UV PC1600).

E. Detection of Intracellular Phosphatase Enzyme Production

The PSB isolates were inoculated in Pikovskaya's broth where tricalcium phosphate (TCP) was replaced with organic source (p-glycerophosphate) and incubated at 28 ℃ for 72h on a rotary incubator shaker (Scigenics Biotech: Model Orbitek 400). The method reported by Balamurugan *et al* (2010) was followed. Briefly, 10 cm^3 of the broth culture was withdrawn, centrifuged at 12,000 g at 4 ℃ for 10 minutes. The supernatant was discarded and the pellets were suspended in 5 cm^3 sterile distilled water. These cells served as enzyme source. One cm^3 of sample was taken in a 50 cm^3 Erlenmeyer flask to which 0.25 cm^3 of toluene was added followed by 4 cm^3 of modified universal buffer (MUB) (pH 6.5 for acid phosphatases and pH 9.0 for alkaline phosphatases) and 1 cm^3 of p- nitrophenyl phosphate (pNPP) solution made in the same buffer. The flasks were swirled for a few seconds, stoppered and placed in an incubator at 37 ℃ and incubated for 1h. 1 cm^3 of 0.5M $CaCl_2$ and 4 cm^3 of 0.5M NaOH were added to the flask, mixed and filtered through a Whatman No.3 filter paper. The intensity of yellow colour developed was measured at 410 nm. Pure p-Nitrophenol (pNP) (Sigma-Aldrich) was used as the standard. The phosphatase activity was expressed as amount of pNP (µg/ml) released/h.

F. Detection of Indole Acetic Acid (IAA) Production by Selected PSB

Bacterial cultures were grown for 72 h at 28 ℃ in Nutrient broth supplemented with 2mg/cm^3 L-tryptophan. Fully grown cultures were centrifuged at 12000g for 10 mins. The supernatant (2 cm^3) was mixed with two drops of orthophosphoric acid and 4 cm^3 of the Salkowski reagent (50 cm^3 35% perchloric acid mixed with 1 cm^3 0.5M $FeCl_3$ solution). Development of pink colour indicated IAA production. Optical density was measured at 530nm with the help of Shimadzu 1650PC spectrophotometer. Concentration of IAA produced by cultures was measured by comparison with a standard graph of IAA (Hi-media) (range of 10–100 µg/cm^3) (Rahman *et al*, 2010, Vikram *et al*, 2007).

G. Determnation of Types of Siderophores

The chrome azurol sulfonate (CAS) assay (Schwyn & Neilands, 1987) was used since it is comprehensive, exceptionally responsive, and most convenient. Cultures were grown in iron deficient succinic acid medium (SAM) containing in (g/L): KH_2PO_4: 6.0, K_2HPO_4: 3.0, $(NH_4)_2SO_4$: 1.0, $MgSO_4.7H_2O$: 0.2, Na-succinate: 4.0, pH 7.0 adjusted using 1N NaOH. Sterile SAM (50 cm^3) in 250 cm^3 Erlenemeyer flask was inoculated with 0.5 cm^3 of the culture suspension and incubated at 28 ℃ for 24-48h at 180 rpm (until turbidity was observed). The broth was centrifuged at 12,500g for 10 min. The cell free supernatant (1 cm^3) was mixed with 1 cm^3 of the CAS reagent. Production of siderophores was indicated by the change in the colour of the CAS reagent from blue to orange.

The types of siderophores were determined by using specific assay, viz. Arnow's test for catecholate type (Arnow, 1937), Csaky test for hydroxamate type siderophore (Csaky, 1948) (Schwyn and Neilands, 1995) and Vogel's test for carboxylates (Murugappan. *et al*, 2006).

H. Identification of PSB

1) Biochemical identification

For preliminary identification of the PSB, the test cultures were subjected a host of different biochemical tests (Table VI).

2) Molecular Identification

16S rRNA sequencing of microbes involved a multi-stage approach beginning with isolation of the genomic DNA of cultures. This DNA was then used as a template for specific amplification of the 16S rRNA region of the DNA by PCR technique using 16S rRNA specific universal primers 27F and 1492R in a MJ Research PTC-225 Peltier Thermal Cycler. The amplified segment of the bacterial DNA was used as the template for performing sequencing using the 16S rRNA specific universal primers 518F and 1492R. ABI 3730xl sequencer (Applied Biosystems) was used for this purpose. Sequence data was aligned and analyzed for identifying the isolate using the BLASTn search tool (Boratyn *et al*, 2013).

III. Results and Discussion

A. Screening and Isolation of Phosphate Solubilizing Bacteria

A total of 134 different colonies were obtained (Table II). These cultures were purified and isolated on Pikovsaya's agar medium and P-solubilization activity was confirmed by replating and observing zones of clearance. The colonies were also studied for their characteristics and Gram nature. The colonies showing a

good solubilization index were selected for further studies and each colony was assigned a unique identification number (Table III).

TABLE II: NUMBER OF PSB ISOLATED FROM DIFFERENT SOURCES.

Sr. No.	Sample and its Source	No. of Isolates
1	Areca Nut Dive Agar, Maharashtra	4
2	Cashew nut Rhizosphere a. Unhavare village, Dapoli, Maharashtra a. Kurumassery village, Aluva, Kerala	 11 13
3	Coconut Rhizosphere b. Unhavare village, Dapoli, Maharashtra c. Muppathadam village, Aluva, Kerala	 9 6
4	Coconut fruit Dive Agar, Maharashtra	4
5	Garlic Rhizosphere Otur Village, Pune, Maharashtra	9
6	Grapes Rhizosphere Otur Village, Pune, Maharashtra	12
7	Jack fruit Rhizosphere Muppathadam village, Aluva, Kerala	6
8	Mango Rhizosphere Muppathadam village, Aluva, Kerala	13
9	Pomegranate Rhizosphere Otur Village, Pune, Maharashtra	6
10	Rice Rhizosphere a. Unhavare village, Dapoli, Maharashtra b. Kurumassery village, Aluva, Kerala	 10 13
11	Spoilt Garlic Bulb Local market, Mumbai	12
12	Inflorescence of *Pandanus fascicularis* Dive Agar, Maharashtra	6
	Total No. of Isolates	**134**

B. Determination of Solubilization Indices of Potential PSB

The primary tool used for scaling down the number of isolates for potential phosphate solubilizing activity was determining the solubilization index. Twenty three of the 134 isolates showed solubilization indices ≥ 1.2 (Table III) and these were selected for further studies.

Several researchers have used solubilization index as a screening tool for selection of potent PSB isolates. It provides a primary quantitative measure of the extent of solubilization of inorganic insoluble phosphate that a culture can induce (Chen, 2008; Monica, 2010). Literature reports SI values ranging from 1.2 to > 2.7 (Ghosh *et al*, 2008). The SI for many of the cultures isolated were at par with the reported values and some were much higher than those reported. There are very few reports (Sarkar *et al*, 2012, P. George *et al*, 2012) from around the world of work related to isolation and characterization of PSB from most of the sources. There is no literature mentioning the use of Pomegranate rhizosphere soil, grapes, and *Pandanus fascicularis* flower for isolation of PSB. Literature indicates that pomegranate orchards in particular have a low requirement for application of P fertilizers (*Growing Pomegranates in California, 1980*); this could probably be because soils in which pomegranate plants are cultivated are naturally abundant with efficient phosphate solubilizing bacteria that reduce the requirement for application of phosphorus as fertilizers.

C. Determination of Phosphorus Solubilizing Efficiency of Isolates

Phosphate solubilizing bacteria have the ability to make unavailable phosphorus available by bringing them into the aqueous solution. This ability can be quantified by measuring the amount of free soluble phosphorus after inoculating and incubating the test cultures in media containing insoluble forms of phosphorus *viz.* tricalcium phosphate, aluminium phosphate, iron phosphate etc. The data presented in Fig. 1 reveals that maximum solubilization of phosphorus was effected by culture no. GN/PSB/PC2 which solubilized ~574 ppm, followed by GN/PSB/KC5 (404 ppm), GN/PSB/KW5 (~401 ppm), and GN/PSB/CC2 (374 ppm). Very few reports have been published that demonstrate the ability of PSB to solubilize inorganic phosphate more than 200 ppm (Chen *et al*, 2006; Banerjee *et al*, 2010, Thakuria *et al*, 2003). Interestingly, while GN/PSB/KC5, KW5, CC2, and TC3 bring about maximum solubilization of phosphorus within 24 hours of inoculation, culture no. GN/PSB/PC2 solubilized phosphorus at a relatively slower rate – maximum solubilization equivalent to approx. 574 ppm is seen at the end of 120h incubation.

Most of the published works indicate that most cultures carry out maximum P solubilization within 24-48h of inoculation (Balamurugan *et al*, 2010; P. George, 2012; Pandey *et al*, 2005). Similarly, phosphate solubilization reported for many of the cultures including standard cultures such as *Bacillus megaterium* (Rodriguez, 1999) is far lesser than our report.

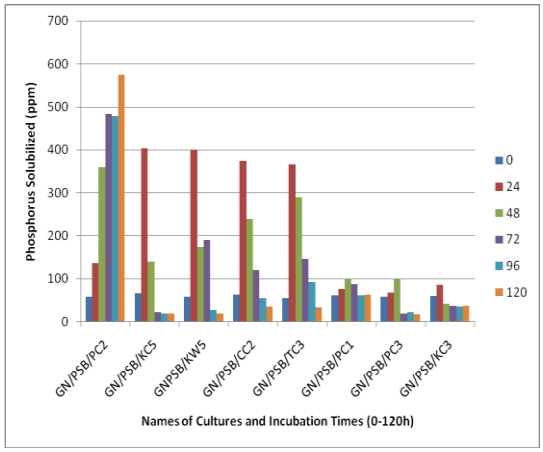

Figure 1. Inorganic phosphorus solubilized by cultures (ppm)

TABLE III: SOLUBILIZATION INDICES OF POTENTIAL PSB ISOLATES AND THEIR SOURCES.

Source	No.	Culture Code	Solubilization Index (SI)
Cashew Nut Rhizosphere	1	GNKC/PSB/KC1	1.5
	2	GNKC/PSB/KC2	1.7
	3	GNKC/PSB/KC3	**3.1**
	4	GNKC/PSB/KC4	1.9
	5	GNKC/PSB/KC5	**4.8**
	6	GNKC/PSB/KC6	2.0
	7	GNKC/PSB/KC7	2.2
	8	GNKC/PSB/KC8	2.2
Coconut Rhizosphere	1	GNKC/PSB/CC1	4.3
	2	GNKC/PSB/CC2	**4.7**
	3	GNKC/PSB/CC3	4.0
	4	GNKC/PSB/CC4	1.2
Pomegranate Rhizosphere	1	GNKC/PSB/PC1	**3.2**
	2	GNKC/PSB/PC2	**3.6**
	3	GNKC/PSB/PC3	2.9
Inflorescence of *Pandanus fascicularis*	1	GNKC/PSB/KW5	**4.3**
Tomato Rhizosphere	1	GNKC/PSB/TC2	2.4
	2	GNKC/PSB/TC3	**4.9**
Garlic Rhizosphere	3	GNKC/PSB/GrC1	1.2
	4	GNKC/PSB/GrC2	1.4
Rice Rhizosphere	1	GNKC/PSB/RC1	1.8
	2	GNKC/PSB/RC2	1.8
	3	GNKC/PSB/RC3	2.3

D. *Detection of Phosphatase Enzyme*

Phosphatase enzyme production was seen only in the samples in which modified universal buffer at pH 6.5 was used indicating that all the 4 positive cultures: GN/PSB/CC2, PC2, KC3, and KC5 (Table IV). produced intracellular acid phosphatases.

TABLE IV: PHOSPHATASE ENZYME PRODUCTION ASSAY

Culture	Assay Result	Type of Phosphatase
GN/PSB/CC2	+	Acid
GN/PSB/KC3	+	Acid
GN/PSB/KC5	+	Acid
GN/PSB/PC2	+	Acid
GN/PSB/KW5	ND	NA
GN/PSB/PC1	ND	NA
GN/PSB/PC3	ND	NA
GN/PSB/TC3	ND	NA

+: positive for phosphatase production; ND: not detected; NA: not applicable

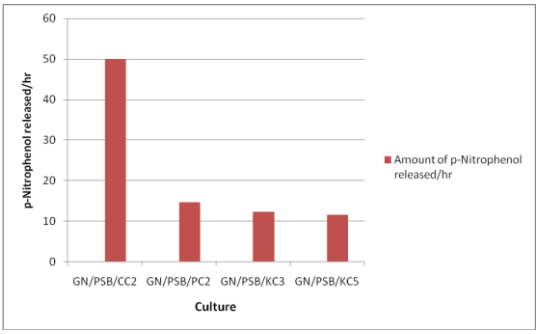

Figure. 2. Phosphatase enzyme produced by PSB isolates

Culture no. GN/PSB/CC2 produced more than 50 µg/cm^3 of pNP/h followed by GN/PSB/PC2 (~14 µg/cm^3), GN/PSB/KC3 (13 µg/cm^3) and GN/PSB/KC5 (11 µg/cm^3) (Fig. 2). The production of acid phosphatase enzyme by these four cultures indicated that these cultures can also solubilize the other major form of insoluble phosphorus present in soils *viz.* organic phosphates.

E. *Detection of Indole Acetic Acid (IAA)*

The assay results presented in Fig. 3 revealed that 3/8 cultures namely, GN/PSB/CC2 (25 ppm), KC3 (22 ppm) and KC5 (19 ppm) were positive. This is consistent with the findings of several researchers who have reported IAA production by different rhizobacteria in the range from 2.0-22.0 ppm (Thakuria *et al*, 2004, Sharma *et al*, 2012).

Many groups of researchers have reported an association of IAA with plant growth promoting rhizobacteria (PGPR) (Spaepan *et al*, 2007; Malhotra *et al*, 2009). It is known that appropriate concentrations of exogenous IAA stimulate the growth and development of plant root systems (Patten and Glick, 2002). It has been widely reported that IAA production is linked to presence of tryptophan. In the present study, IAA production by the PSB isolates was observed only when tryptophan was added to the culture medium. Tropical soils are extremely rich in polyphenols and humic acids that often cut down on the availability of phosphorus and trace elements to plants thereby inhibiting their growth (Rahman et al, 2010; Andriesse, 1988). It is postulated that PGPR are capable of degrading such polyphenols and humic

substances and thus can be important for the plants inhabiting the ecosystem (Rahman *et al*, 2010). While it is known that plants exude very low amounts of tryptophan into the rhizosphere, the polyphenols and humic substances can act as a good source of tryptophan for the PGPR that can utilize the tryptophan to produce IAA (Rahman *et al*, 2010). Thus, the results obtained for the production of IAA by the PSB isolates indicate that some of these cultures can probably play an important role in plant growth promotion. This plant growth promotion would be in addition to the role played by these cultures in improving availability of soluble phosphates to the plants.

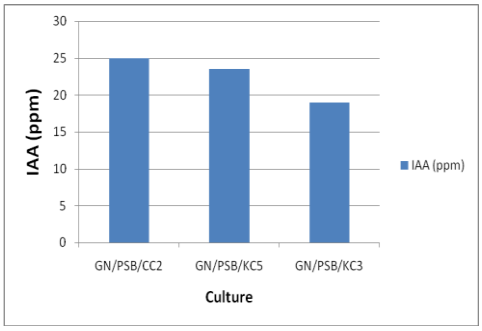

Figure. 3. Indole acetic acid production by PSB isolates

F. Detection of Types of Siderophore

Microorganisms growing under aerobic conditions need iron for a variety of functions including reduction of oxygen for synthesis of ATP, reduction of ribonucleotide precursors of DNA, for formation of heme, and for other essential purposes. A level of at least 1 µM Fe is needed for optimum growth. These environmental restrictions and biological imperatives have required that microorganisms form specific molecules that can compete effectively with hydroxyl ion for the ferric state of iron, a nutrient which is abundant but essentially unavailable. The CAS assay revealed that cultures GN/PSB/CC2, KC5, KC3 and PC2 produced iron chelating siderophores. The results of the assays (Table V) showed that cultures CC2 and KC5 produced catecholates; KC3 showed production of hydroxamates whereas PC2 tested positive for carboxylates.

TABLE V: SIDEROPHORE PRODUCTION BY PSB ISOLATES.

Culture	Assay Result	Type of Siderophore
GN/PSB/CC2	+	Catecholate
GN/PSB/KC3	+	Hydroxamate
GN/PSB/KC5	+	Catecholate
GN/PSB/KW5	+	Catecholate
GN/PSB/PC2	+	Carboxylate
GN/PSB/PC1	ND	NA
GN/PSB/PC3	ND	NA
GN/PSB/TC3	ND	NA

+ : positive for siderophore production ND: not detected; NA: not applicable

Culture PC2 produces siderophores in addition to solubilization of organic as well as inorganic phosphorus; cultures CC2 and KC3 have demonstrated the ability to produce plant beneficial hormones along with siderophore production and phosphate solubilization. These attributes make these three cultures namely, PC2, CC2 and KC3, excellent candidates for evaluation as members of the phosphate solubilizing microbial consortia.

G. Identification of PSB Using Biochemical and Molecular Techniques

Biochemical tests performed for the PSB isolates led to their probable identification up to genus level. Results for some of the common tests performed are listed in Table VI. in addition to biochemical testing the cultures were also identified using the modern 16S rRNA technique. The 16S rRNA sequences obtained, following the sequencing reactions set up as described previously, were analyzed using the BLASTn sequence analysis tool; the results are shown in Table VII. Results for 16S rRNA identification matched with those for biochemical testing for some of the isolates. Biochemical identification provides primary information regarding the probable genus level classification of isolates prior to their confirmed identification using 16S rRNA technique.

The data presented in Table VII shows that cultures GN/PSB/CC2, KC5, KW5, and TC3 were identified as *Burkholderia* spp. Literature survey showed that members of this species are proven to be effective as phosphate solubilizing bacteria (Song, 2008; Rodriguez 1999) and as producers of plant growth promoting factors. Mellado *et al* (2007) have reported that *Burkholderia tropica* and other members of the genus exhibited activities involved in nitrogen fixation, bioremediation, plant growth promotion, or biological control *in vitro*.

Culture GN/PSB/KC3 was identified to be *Agrobacterium tumfaciens (*recently reclassified as *Rhizobium radiobacter)* (http://www.uniprot.org/taxonomy/358). There is only one report from China till date regarding the phosphate solubilizing potential of *Rhizobium radiobacter* (Hao *et al*, 2012). These results are in support of this finding confirming the phosphate solubilization activity of *Rhizobium radiobacter*. Members of this genus are also proven nitrogen fixers thereby adding their value to the consortium as plant growth promoting rhizobacteria. This is the first ever report regarding IAA and siderophore production by *Rhizobium radiobacter*.

Further, culture no. GN/PSB/PC2 was identified as *Ralstonia pickettii*. While *Ralstonia* spp. are proven efficient N_2 fixers (Kuklinsky *et al*, 2004 and Bulut, 2013), this is the first ever report of effective phosphate solubilization by *Ralstonia pickettii* culture. They are known to be producers of IAA (Kulinsky *et al*, 2004), however siderophore production by these cultures was previously unknown. This is a very important finding as both the source from which this culture was isolated (pomegranate rhizosphere) and also the culture itself are new findings in the field of phosphate solubilization research.

TABLE VI: RESULTS FOR BIOCHEMICAL IDENTIFICATION TESTS

Category	Test	GN/PSB/ CC2	GN/PSB/ KC3	GN/PSB/ KC5	GN/PSB/ KW5	GN/PSB/ PC1	GN/PSB/ PC2	GN/PSB/ PC3	GN/PS B/ TC3
Sugar Fermentation Tests	D-Glucose	+	+ with gas	-	-	+	-	-	-
	D-Maltose	-	+	-	-	-	+ with gas	+ with gas	-
	D-Mannose	-	+	-	-	-	NA	NA	NA
	D-Sucrose	-	-	-	-	+	-	+	+
	D-Xylose	-	-	-	-	-	-	-	-
	D-Lactose	-	+	+	+	+	-	-	-
	D-Trehalose	-	+	+	+	+	NA	NA	NA
	D-Mannitol	-	+	-	-	-	-	+	-
	D-Sorbitol	-	-	-	-	-	NA	NA	NA
Enzyme Production assays	Dehydrogenase Production	+	+	+	+	+	+	+	+
	Oxidase production	+	+	-	+	+	+	-	-
	Indole production	-	-	-	-	-	-	-	-
	Catalase Production	+	+	+	+	+	+	-	-
	Urease Production	-	-	+	+	-	+	-	-
	Lipase Production	-	-	-	-	-	NA	NA	NA
Other Biochemical tests	Citrate Utilization	+	+	+	+	+	+	-	+
	Malonate Utilization	+	+	+	+	+	+	-	+
	Nitrate Reduction	-	+	+	+	-	-	-	+
	Methyl Red Test	-	-	-	-	-	-	-	-
	Voges-Proskauer Test	-	-	-	-	-	-	-	-
	Triple Sugar Iron Test	k/k	k/a with gas	k/k with gas	k/k with gas	k/k with gas	k/a with gas	gas	k/k with gas
Gram Nature and Morphology		Gram Negative short rods	Gram Negative rods	Gram Negative short rods	Gram Negative short rods	Gram Positive oval cells	Gram Negative rods in chains	Gram Negative thick rods	Gram Negative short rods
Probable Identifica-tion		*Burkholderia cepacia*	*Enterococcus gallinarium*	*Burkholderia sp.*	*Burkholderia sp.*	*Candida tropicalis*	*Pseudomonas* sp	*Pseudomonas* sp.	*Pseudomonas* sp.

Key:+ = positive; - = negative (for all tests)
For TSI test: a = acid; k = alkaline; k/a = "K over A" or "alkaline over acid" and refers to an alkaline slant and acid butt; k/k = alkaline slant and alkaline butt

TABLE VII: IDENTIFICATION OF PSB ISOLATES BY 16S RRNA SEQUENCING

Name of the Culture and source	Culture Identity	Percentage Homology
GN/PSB/CC2 Coconut rhizosphere (Maharashtra)	*Burkholderia cepacia*	≥99%
GN/PSB/KC3 Cashew nut rhizosphere (Maharashtra)	*Agrobacterium tumefaciens* (updated scientific name: *Rhizobium radiobacter*)	≥99%
GNPSB/KC5 Cashew nut rhizosphere (Kerala)	*Burkholderia tropica*	≥99%
GN/PSB/KW5 Inflorescence of *Pandanus fascicularis* (Maharashtra)	*Burkholderia tropica*	≥99%
GN/PSB/PC1 Pomegranate Rhizosphere (Maharashtra)	*Candida tropicalis*	≥99%
GN/PSB/PC2 Pomegranate Rhizosphere (Maharashtra)	*Ralstonia pickettii*	≥99%
GN/PSB/PC3 Pomegranate Rhizosphere (Maharashtra)	*Ochrobactrum anthropi*	≥99%
GN/PSB/TC3 Tomato Rhizosphere (Maharashtra)	*Burkholderia tropica*	≥99%

Ochrobactrum anthropi (culture no. GN/PSB/PC3) is an important plant growth promoting rhizobacterium (PGPR) previously reported to be capable of phosphate solubilization (Chakraborthy *et al*, 2009) in addition to production of siderophores, IAA and plant phenolics. Also, certain reports suggest that *Ochrobactrum anthropi* exhibits antifungal activity against several known plant pathogens (Chakraborthy *et al*, 2009). These attributes are highly desirable in a culture for use as a PGPR.

Culture no. GN/PSB/PC1 was identified as *Candida tropicalis.* Amprayn *et al* (2012) reported that *Candida tropicalis* was able to stimulate rice seedling growth. Additionally they also reported the production of small quantities of IAA and good ACC-deaminase by the culture. Although soils are known to contain yeasts, little is known about their ecology and the role which they play in mineral cycling. Data on phosphate solubilization in vitro by soil yeasts are scarce, although such results would be useful as an essential prerequisite to determining the role of soil yeasts in mediating this transformation in soil (Al-Falih, 2005).

IV. Conclusion

The work presented here provides valuable information regarding isolation of phosphate solubilizing bacteria from novel sources. The ability of *R. picketti* as a PSB has not been previously reported. Further, this is the first report regarding use of pomegranate rhizosphere as a source for screening of phosphate solubilizing bacteria. GN/PSB/KW5 isolated from *Pandanus fascicularis* inflorescence is also a novel finding. These results are encouraging as one can explore even more unconventional sources for isolation of variety of potent PSB.

The isolated cultures have been proven to be potentially efficient PGPR capable of promoting plant growth through multiple approaches *viz.* phosphate solubilization (organic as well as inorganic), IAA production, siderophore production, and possibly other reported plant growth promoting attributes such as nitrogen fixation, phytase enzyme production, ACC-deaminase activity and pathogen inhibition. Many of the cultures studied here have superior ability to solubilize inorganic phosphorus as compared with those previously reported by others.

Our preliminary findings indicate that these cultures are potentially good members of a microbial consortium that can solubilize soil phosphorus efficiently in a sustained manner. The macro and microenvironments prevailing in soil are quite dynamic and vary widely. It is difficult to understand the variations in soils in the absence of knowledge derived from both chemical and biological approaches, because microorganisms affect the environment and vice versa. Despite their small volume, soil microorganisms are key players in the global cycling of organic matter, reworking organic residues or mineralizing them to CO_2, H_2O, nitrogen, phosphorus, sulfur, and other nutrients. A consortium of the cultures reported here would probably be able to provide sustained and controlled release of inorganic phosphorus over a longer period of time. As reported in our results, some isolates show sustained solubilization over a long period of time. Therefore, consortium of cultures having faster and slower rate of utilization of phosphate would make it a sustained viable process.

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