3.1 INTRODUCTION

Phosphorus (P) is one of the major essential macronutrients for biological growth and development [Ehrlich, 1990]. Deficiency of phosphorus is one of the important chemical factors restricting plant growth. Phosphorus contributes remarkably to photosynthesis, energy and sugar production, nucleic acid synthesis, and promotes N₂ fixation in legumes [Saber et al., 2005]. Though soil constitutes 0.5% phosphorus, only a minute amount is available for plant absorption, others remain as insoluble salts and cannot be absorbed by plants [Rodriguez & Fraga, 1999]. Besides this, a large portion of chemical fertilizers with high phosphorus content applied to soil is immobilized rapidly and becomes unavailable to plants [Goldstein, 1986]. Phosphorus is added to soil in the form of phosphatic fertilizers, part of which is utilized by plants and the remaining converted into insoluble fixed forms. A greater part of soil phosphorus, approximately 95 to 99% is present in the form of insoluble phosphates and hence cannot be utilized by the plants [Vassileva et al., 1998].

Phosphate solubilizing microorganisms (PSMs) play an important role in supplementing phosphorus to the plants, allowing a sustainable use of phosphate fertilizers. Application of PSMs in the field has been reported to increase crop yield. Several mechanisms like lowering of pH by acid production, ion chelation and exchange reactions in the growth environment have been reported to play a role in phosphate solubilization by PSMs [Abd-Alla, 1994; Whitelaw, 2000]. Species of Aspergillus, Penicillium and yeast have been widely reported to solubilize various forms of inorganic phosphates [Whitelaw, 2000]. Siderophores are low molecular weight, extracellular compounds with a high affinity for ferric iron, that are secreted by microorganisms to take up iron from the environment
[Hofte, 1993] and their mode of action in suppression of disease was thought to be solely based on competition for iron with the pathogen [Bakker et al., 1993; Duijff et al., 1997]. Fluorescent *Pseudomonas* characterized by the production of yellow-green pigment termed pyoverdines which fluoresce under UV light and function as siderophores [Demange et al., 1987]. The role of siderophores produced by PGPR in plant growth promotion was first reported by Kloepper et al. (1981). The siderophores were later reported to be implicated in the suppression of plant pathogens [Bakker et al., 1986; Becker & Cook, 1988].

Competition for iron between pathogens and siderophores of PGPR has been implicated in the biocontrol of wilt diseases caused by *Fusarium oxysporum* [Kloepper et al., 1980; Scheer & Bakker, 1982], damping off of cotton caused by *Pythium ultimum* [Loper, 1988] and *pythium* root rot of wheat [Becker & Cook, 1988]. Rhizobacteria produce various types of siderophores (Pseudobactin & ferroxoxamine B) that chelate the scarcely available iron and there by prevent pathogens from acquiring iron [Loper & Buyer, 1991]. Several strains of siderophore producing *Bacillus* and *Pseudomonas* have been shown to inhibit *F. oxysporum* f. sp. *cubense*, *F. oxysporum* f. sp. *vasinfectum*, *Rhizoctonia solani* and *Acrocylindrium oryzae* [Sakthivel et al., 1986]. Though siderophores are part of primary metabolism (iron is an essential element), on occasions it also behave as antibiotics which are commonly considered to be secondary metabolites [Haas & Defago, 2005]. Ultimately this led to iron starvation and prevented the survival of the microorganisms including nematodes.

Plant growth promoting rhizobacteria effects exerted by some plant-beneficial bacteria are due to the bacterial production of plant hormones, such as, indole-3-acetic acid (IAA), cytokinins and gibberellins [Bloemberg & Lugtenberg, 2001; Bottiniet al., 2004]. One of the direct mechanisms by which PGPR promote plant growth is by production of plant growth regulators or phytohormones [Glick, 1995]. Frankenberger and Arshad, (1995) have discussed in detail the role of auxins, cytokinins, gibberellins, ethylene and abscisic acids (ABA) which, when applied to plants, help in increasing plant yield and growth. Microbial production of individual phytohormones such as auxins and cytokinins
has been reviewed by various authors over the last 20 years [Pilet et al., 1979; Hartmann et al., 1983; Fallik & Okon, 1989; Barbieri & Galli, 1993; Patten & Glick, 1996; Patten & Glick, 2002]. The physiological effect of microbial IAA on plant growth depends ultimately on the amount of hormone that is available to the plant, which is based on the interaction between the plant and the bacterium [Patten & Glick, 1996]. Three general types of possible association between the plant and the bacterium are important in order to exert a positive effect. In the case of beneficial rhizobacteria the effect is primarily thought to be advantageous when the bacteria are colonizing the external surface of the plant [Del Gallo & Fendrik, 1994]. Early work showed that PGPR such as Azotobacter paspali secreted IAA into culture media and significantly increased the dry weight of leaves and roots of several plant species following root treatment [Barea & Brown, 1974]. Azospirillum brasilense, 19 which had the ability to produce plant growth-promoting substances such as indole acetic acid, indole lactic acid, gibberellin and cytokinin when applied to pearl millet (Pennisetum americanum L.), increased the number of lateral roots which were densely covered by root hairs [Tien et al., 1979].

IAA was detected in 80 per cent of bacteria isolated from the rhizosphere [Loper & Schroth, 1986]. Growth substances Indole acetic acid (IAA) and gibberellins (GA₃) were isolated from cultures of Azotobacter spp which altered growth of stems, leaves and flowers of peas and tomatoes. Further Brown and Burlinghan, (1968) compared effects of treating tomatoes with GA₃ and with cultures of Azot. chroococcum producing small amounts of GA₃ and IAA. These workers supported the hypothesis that PGPR produced growth factors which were in sufficient quantity in the inoculum added to alter plant development.

The enhancement of plant growth by members of bacilli strains, such as Bacillus and Paenibacillus, has been well documented [Mc Spadden & Gardener, 2004]. They promote plant growth by a number of mechanisms, including production of phytohormones, such as indole acetic acid (IAA) [Choudhary & Johri, 2009]. Phytohormones such as IAA may indirectly improve Phosphorous acquisition by plants by increasing root growth [Marschner et al., 2011]. The
PGPR strains of *Pseudomonas* are known to produce IAA and GA$_3$ in the rhizosphere of plants and stimulate the crop growth [Kloepper & Scroth, 1978]. Rhizosphere colonizing fluorescent *Pseudomonas* significantly increased growth and yield of crops [Suslow & Schrot, 1982].

Phosphate solubilization by PGPR is another important property as non-availability of phosphate can be grown limiting for plants. PGPR could solubilize minerals, source of complex phosphate as well as release phosphate from organic sources via two phosphatase enzymes. Mineral phosphate solubilization in bacteria occurs by production of organic acids and organic phosphate release is aided by acid and alkaline phosphatases [Rodriguez & Fraga, 1999]. Phosphatase producing bacteria are common in the rhizosphere [Nautiyal *et al.*, 2000] and secretion of organic acids and phosphatase are common method of facilitating the conversion of insoluble forms of P to plant available forms [Kim *et al.*, 1998]. The solubilization of P in the rhizosphere is the most common mode of action implicated in PGPR that increase nutrient availability to host plants [Richardson, 2001]. More importantly, increases in root length and root surface area are sometimes reported [Galleguillos *et al.*, 2000; German *et al.*, 2000; Jacoud *et al.*, 1999; Volkmar and Bremer, 1998]. Fallik *et al.*, (1994) reported that inoculation of maize with *Azospirillum brasilense* resulted in a proliferation of root hairs which could have dramatic effects on increasing root surface area.

Fungal plant pathogens are among the most important factors that cause serious losses to agricultural products annually [Ekundayo *et al.*, 2011]. Management of fungal diseases using antagonistic microorganisms, known as biological control, has been the focus of intense research worldwide [Killani *et al.*, 2011]. Biological control of plant pathogens is considered as a viable alternative method to chemical control. Nonpathogenic soil bacteria, the plant growth promoting rhizobacteria (PGPR) living in association with roots of higher plants enhance the adaptive potential of the hosts and increase their growth through a number of mechanisms [Gholami *et al.*, 2009; Zongzheng *et al.*, 2009; Heydari & Pessarakli, 2010; Killani *et al.*, 2011; Farhana *et al.*, 2011; Saharan & Nehra, 2011].
3.2 MATERIALS AND METHODS

**Inoculum Preparation:** Bacterial isolates were grown in tubes containing 3 ml Nutrient broth (NB) and incubated overnight at 30°C on shaker (120 rpm). Cultures were centrifuged at 10,000 rpm for 10 min and washed with 1 ml sterile 0.85% NaCl to remove free Pi present in medium. The bacterial pellet was re-suspended in 1 ml of 0.85% NaCl and was used as inoculum in the experiments.

**Organic Acid Analysis:** 50 ml NB in 250 ml Erlenmeyer flask was inoculated (1%) and incubated at 30°C for 72 h on shaker (120 rpm). Culture supernatants were filtered through 0.2 µm nylon filter and 20 µl samples were injected in the HPLC instrument (Shimadzu) equipped with C18 reverse phase silica column. Mobile phase consisted of 5 mM H2SO4 and the flow rate was 1 ml/min and oven temperature was maintained at 40°C. Organic acids were detected by monitoring at A210. Solutions of pure organic acids (gluconic, acetic, pyruvic, lactic, citric and succinic) were separately injected and their peak retention times were compared with that of the samples.

**Antifungal Activity:** The *in vitro* inhibitions of mycelium growth of *Aspergillus flavus, Aspergillus niger, Alternaria solani, Fuzarium oxysporum, Penicillium roqueferti* and *Rhizoctonia solani* by the PGPR were tested on solid agar media [Caten & Jinks, 1968]. For each bacterial isolates PGPR suspension was streaked on the margin PDA media plates and a 4 mm agar disc of respective plant pathogenic fungi from fresh PDA culture was placed at the other marginal side and incubated at 25 ± 2°C for seven days. Control plates were inoculated only by fungi. The radius of the fungal colony towards and away from the bacterial colony were noted. The antifungal index was calculated using the following calculation.

\[
\text{Antifungal index} = \left[ \frac{(R - r)}{R} \times 100 \right]
\]

Where, \( r \) is the radius of the fungal colony opposite the bacterial colony and \( R \) is the maximum radius of the fungal colony away from the bacterial colony.
**Acid and Alkaline Phosphatase Activity:** This was estimated by measuring the production of either acid or alkaline phosphatases by all PGPR isolates [Tabatabai & Bremner, 1969]. The strains were grown in NB at 30°C. The bacterial cultures were filtered through Whatman filter paper and homogenized using 0.02 M Tris buffer (pH 7.5). The macerate was centrifuged at 10,000 rpm for 20 min and the supernatant was collected. For assaying alkaline phosphatase activity 0.1 ml of the culture supernatant were added to a reaction mixture consisting of 0.5 ml of a Tris citrate buffer (pH 8.5), 0.1 ml of MgCl₂ (0.1 M) and 1 ml of p-Nitrophenol phosphate (1 mg/ml) solution. The same procedure was followed for acid phosphatase activity but an acetate buffer (pH 4.5) was used instead of the Tris citrate buffer. The test tubes were incubated at 30°C for 30 min. After that, 5 ml of 0.5 M NaOH were added and the p-Nitrophenol released was measured by spectrophotometry at 405 nm. The measured values were converted to micromoles of p-Nitrophenol with reference to a standard curve. One enzyme unit was defined as the amount of enzyme that catalysed formation of 1 mol of end product (p-Nitrophenol) in 1 min under experimental conditions.

**Quantitative Phosphate Solubilization:** Quantitative estimation of P solubilization was carried out, by inoculating 1 ml of bacterial suspension (3 × 10⁷ cfu/ml) in 50 ml of National Botanical Research Institute Phosphate (NBRIP) broth [Nautiyal, 1999], in 150 ml Erlenmeyer flasks, and incubated for 7 days on shaker (120 rpm) at 30°C. Cultures were then centrifuged at 10,000 rpm for 10 minutes, and the Pi content in the supernatant was spectrophotometrically estimated by Vanado-molybdate method [Gulati et al., 2008], and pH of the medium was determined.

**Indole Acetic Acid Production:** Indole acetic acid produced by bacteria was determined as described by Brick et al. [Brick et al., 1991]. Bacterial cultures were grown in NBRIP broth amended with tryptophan (100 µg/ml) at 30°C for 48 h on shaker (120 rpm). The cultures were centrifuged at 3000 rpm for 30 minutes. 2 ml supernatant was mixed with two drops of o-phosphoric acid and 4 ml of Salkowski reagent (50 ml, 35% of perchloric acid, 1 ml 0.5 M FeCl₃ solution).
Development of pink color indicated IAA production and $A_{530}$ was recorded. This experiment was carried out at different incubation time (Day 1-6), pH (4-8), temperature (30, 37 and 45°C), using different carbon source (glucose, galactose, sucrose, lactose and maltose) and at varying tryptophan concentrations (0, 5, 10, 25 µg/ml).

**Siderophore Production Assay:** From overnight grown culture, 10 µL cell suspension was inoculated onto NB (pH-7.2) supplemented with iron (10 µmol/L FeCl$_3$) and tryptophan (10 µmol/L) without any exogenous amino acids. After 48 h of incubation, the bacterial cells were pelleted by centrifugation at 4000 rpm for 15 min. Absorbance spectra were determined from 200 to 800 nm using UV-Visible spectrophotometer. Siderophore production by PGPR isolates was determined by using the method of Van Peer *et al.* (1990). A reference was prepared using uninoculated NB. Both the test and reference were read at 380 nm and percent Siderophore units in the culture were determined using following formula.

\[
\text{Percent Siderophore units} = \left(\frac{A_R - A_S}{A_R}\right) \times 100
\]

Where $A_R$=Absorbance of reference at 380 nm and $A_S$=Absorbance of test sample at 380 nm.

**Gibberellins Production:** Cultures of PGPR inoculated into the NB and NBRIP medium and incubated for 72 h at 30°C. The method of Holbrook *et al.* with slight modifications was used for the determination of gibberllins. To 15 ml of supernatant, 2 ml of zinc acetate reagent (21.9 g zinc acetate + 1 ml of glacial acetic acid and volume was made up to 100 ml with distilled water) was added. After 2 minutes, 2 ml of potassium ferrocyanide (10.6% in distilled water) was added and was centrifuged at low speed (4000 rpm) for 15 minutes. To 5 ml of supernatant 5 ml of 30% HCl was added and the mixture was incubated at 20°C for 75 min. For blank 5 ml of 5% HCl was used. Absorbance was read at 254 nm.
concentration of gibberellins was calculated by preparing standard curve by using gibberellic acid (GA$_3$) as standard (100-1000 g/ml).

**Acid and Alkaline phosphatase Activity:** Acid and alkaline phosphatase was assayed as Tabatabai and Bremner (1969). Exact 3 ml of aliquot (48 h grown culture), 1 ml modified Tris-Cl buffer and 1 ml 0.115 M p-NPP were pipetted into a 20 ml reagent vial. The mixture was incubated at 37°C for 1 h. Phosphatase reaction was stopped by the addition of 20 ml 2 N NaOH. The mixture was transferred to a 50 ml volumetric flask and the volume was made up to with distilled water. The absorbance (yellow colour intensity) was read with a spectrophotometer at 405 nm along with standards which were prepared by using 20 g/ml p-PNP.

**Biocompatibility Assay:** Cultures were grown in tubes containing sterile 4 ml NB for 48 hrs and streaked over the NA perpendicular to each other. Plates were incubated at 30°C for 72 h. After incubation plates were checked for the appearance of zone of inhibition.

**Evaluation of Pathogenicity:** Cultures were spot inoculated over the surface of blood agar and incubated at 37°C for the 72 h. After incubation period plates were visually checked for the presence of haemolytic zones.

3.3 RESULTS

**Organic Acid Analysis by HPLC:** The organic acid analysis of culture supernatant produced by the cultures T-2, T-4, BR-1 and RR-1 showed presence of organic acids (Table 3.1). T-2 did not produced succinic acid while strains T-4, BR-1 and RR-1 did not exhibit production of pyruvic acid. The chromatogram of standard organic acids and test cultures are shown in Fig 3.1-3.4.

**Antifungal Activity:** PDA plates were co-inoculated with plant pathogenic fungal strains and PGPR isolates having multiple PGP activities, after the incubation period isolates showed zone of inhibition against fungi as shown in Fig. 3.5 and Fig. 3.6.
Table 3.1 Production of organic acids by the agitated cultures of PGPR isolates growing on NB at 30°C after 72 h

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Organic acids produced</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em> T-2</td>
<td>Gluconic acid</td>
</tr>
<tr>
<td></td>
<td>Pyruvic acid</td>
</tr>
<tr>
<td></td>
<td>Lactic acid</td>
</tr>
<tr>
<td></td>
<td>Citric acid</td>
</tr>
<tr>
<td><em>K. pneumoniae</em> T-4</td>
<td>Gluconic acid</td>
</tr>
<tr>
<td></td>
<td>Lactic acid</td>
</tr>
<tr>
<td></td>
<td>Citric acid</td>
</tr>
<tr>
<td></td>
<td>Succinic acid</td>
</tr>
<tr>
<td><em>C. malonaticus</em> BR-1</td>
<td>Gluconic acid</td>
</tr>
<tr>
<td></td>
<td>Lactic acid</td>
</tr>
<tr>
<td></td>
<td>Citric acid</td>
</tr>
<tr>
<td></td>
<td>Succinic acid</td>
</tr>
<tr>
<td><em>B. subtilis</em> RR-1</td>
<td>Gluconic acid</td>
</tr>
<tr>
<td></td>
<td>Pyruvic acid</td>
</tr>
<tr>
<td></td>
<td>Citric acid</td>
</tr>
</tbody>
</table>

Inhibition of the growth of (a) *Aspergillus flavus* (b) *Aspergillus niger* (c) *Alternaria solani* (d) *Fuzarium oxysporum* (e) *Penicillium roqueforti* (f) *Rhizoctonia solani* by the PGPR isolates growing on PDA plates.

All the isolates showed lower inhibition index against *R. solani* while isolate T-2 exhibited antifungal index against *A. flavus, A. solani, F. oxysporum* and *Pn. roqueforti* 60, 67, 71 and 73% respectively, While It showed 75% against *A. niger*.

Isolate T-4 inhibited the growth of *Pn. roqueforti* (87%), *A. flavus* (73%), *A. niger* (86%), *A. solani* (76%), *F. oxysporum* (69%) and *R. solani* (2%).
Fig. 3.1: HPLC Chromatograms of standard organic acids

- Acetic acid
- Citric acid
- Gluconic acid
Fig. 3.2: HPLC Chromatograms of standard organic acids

- Lactic acid
- Pyruvic acid
- Succinic acid
Fig. 3.3: HPLC chromatograms of (A) Mixures Standard and supernatants of 72 h grown cultures of PGPR strains (B) T-2 (C) T-4 (D) BR-1 (E) RR-1 growing in NBRIP medium.
BR-1 showed antifungal index 80% against *Pen. roqueforti* while isolate RR-1 showed a comparatively higher antifungal index against all the test plant pathogens except *R. solani*.

Antifungal index against various plant pathogenic fungi is shown Figure 3.7.
Fig. 3.5: Antifungal activity of PGPR isolates against *Aspergillus flavus*, *Aspergillus niger* and *Alternaria solani* on potato dextrose agar plates incubated for 6 days at 30°C.
Fig. 3.6: Antifungal activity of PGPR isolates against *Fuzarium*, *Penicillium roqueforti* and *Rhizoctonia solani* on potato dextrose agar plates incubated for 6 days at 30°C

Control – *Fuzarium oxysporum*  Co-inoculated with PGPR isolates

Control – *Penicillium roqueforti*  Co-inoculated with PGPR isolates

Control – *Rhizoctonia solani*  Co-inoculated with PGPR isolates
Phosphate Solubilization: Strong correlation was observed between the decrease in pH of the medium and phosphate solubilizing activity (Fig 3.8). Phosphate solubilizing activity increased as the pH of the culture medium decreased. pH of the medium after 2 days in the case of the isolate BR-1 and after 4 days with the other three cultures stabilized around 3. The phosphate solubilization also became steady. Four PGPR isolates RR-1n showed higher phosphate solubilization (384 µg/ml). Isolate T-2 solubilized phosphate 365 µg/ml after 6 days of incubation in NBRIP broth. Isolate T-4 showed increasing phosphate solubilization up to 5 days 405 µg/ml but becomes steady after 5 days. BR-1 solubilized 362 µg/ml of inorganic phosphate simultaneously with the decrease in the pH (Figure 3.8).
**IAA Production:** IAA production by selected PGPR strains at varying pH, temperature, incubation time and at different concentration of tryptophan showed that the isolate produced greater amount of IAA at pH 7 while under acidic conditions IAA production decreased (Fig. 3.9). IAA production at different temperature showed increased production at 37°C. All the isolates produced IAA at 45°C but in lower amount as compared with IAA production at 37°C.

Increasing tryptophan concentration showed increase in the IAA production isolate RR-1 produced 20.12 µg/ml of IAA which is higher as compared to other isolates. Study of IAA production at varying incubation time revealed that of IAA production increased observed up to 3 days and decreased then after.

**Siderophore Production Assay:** Siderophore production by isolates followed the order BR-1>T-2>RR-1>T-4. All the isolates produced siderophores after incubation of 72 h in NBRIP broth Fig 3.10.

**Gibberellins Production:** Among the four isolates isolate RR-1 produced 27.8 µg/ml while T-2 (20 µg/ml), T-4 (17 µg/ml) and BR-1 (15 µg/ml) produced in decreasing manner. Fig 3.10.

**Acid and Alkline Phosphatase Activity:** All selected PGPR isolates produced higher alkaline phosphatase activity compared to acid phosphatase. In case of production of acid phosphatase all the strains showed production in range between 39-43 IU/ml.

While for the alkaline phosphatase isolates showed diverse pattern of production in which RR-1 produced 52 IU/ml. In case of isolate T-2, T-4, BR-1 produced 48, 51 and 42 IU/ml respectively (Fig 3.11).

**Biocompatibility Assay:** Isolates when streaked perpendicular to each other (A-E) did not inhibit the growth of other PGPR isolates exhibiting biocompatibility (Figure 3.12).
Fig. 3.8: Changes in the pH (○) and amount of phosphate solubilized (△) by the PGPR strains (A) T-2, (B) T-4, (C) BR-1 and (D) RR-1 during their growth in NBRIP medium. Values are means of three replicates of three independent experiments.

Evaluation of Pathogenicity: To evaluate pathogenicity of selected PGPR isolates were streaked over the surface of blood agar and incubated for 72 h at 30°C. After incubation no haemolytic zones were observed indicated the isolates were non pathogenic to animal and can be used as bio-inoculants (Figure 3.13).
Fig. 3.9: Production of IAA by the agitated cultures of PGPR isolates growing in NBRIP media with varying (a) pH (b) temperature (c) tryptophan concentration (d) incubation time

![Graphs showing production of IAA under varying conditions](image)

Fig. 3.10: Production of (A) Siderophore & (B) Gibberellins by PGPR isolates growing in shake flask cultures of PGPR strains in the NB at 30°C

![Graphs showing production of siderophores and gibberellins](image)
Fig. 3.11: Acid and Alkline phosphatase Activity of PGPR isolates growing in NB after 48 h at 30°C in shaking condition (120 rpm)

![Bar graph showing acid and alkaline phosphatase activity of PGPR isolates.](image)

- Acid phosphatase
- Alkaline phosphatase

Fig. 3.12: Biocompatibility Assay of PGPR isolates

![Biocompatibility assay images of PGPR isolates.](image)
3.4 DISCUSSION

This study reports the characterization of plant growth promoting rhizobacteria having multiple plant growth promoting activity. Microbial strains represent the four different genera like *Pseudomonas*, *Klebsiella*, *Cronobacter* and *Bacillus* showed phosphate solubilization, IAA production, Organic acid production, Antifungal activity, Siderophore production, Gibberellin production and production of acid and alkaline phosphatase along with biocompatibility and non pathogenicity. Isolates showed all the multiple PGPR traits which may lead to growth of plants under indigenous conditions.

Initially all the isolates were checked for their ability to solubilize phosphate. Phosphate solubilization trait was exhibited by the all bacterial strain in which isolate *Klebsiella pneumoniae* T-4 solubilized highest (405 g/ml) in organic phosphate among the selected microbial strains. Phosphate solubilization
showed strong relationship with the decrease in the pH similar results were obtained by the

From the results of this study, it is being reaffirmed that the phosphate solubilization by different PSBs is involved with the production of organic acids [Halder et al., 1990; Goldstein, 1995; Kim et al., 1998; Rashid et al., 2004]. The inverse relationship observed between the pH and soluble-Pi concentration indicates that organic acid production by these PSB strains plays a significant role in the acidification of the medium facilitating the P solubilization. Similar inverse relationship between pH and soluble phosphate was reported earlier by Illmer & Schinner (1995) and Hwangbo et al. (2003). Although the HPLC system that was used here to analyze the organic acids was able to detect different organic acids from the fermented broth supernatants, none of our isolates showed the release of unknown acid instead, Cronobacter malonicicus BR-1 produced Gluconic acid, lactic acid, citric acid and succinic acid reported first time to having role in inorganic phosphate solubilization [Bhatt & Vyas 2014]. Instead of secreting the only one type of organic acid the production of multiple organic acids may have proficient effect over the mineral phosphate solubilization (MPS), Similar observations were recorded by Kim et al. (1997b), wherein genomic DNA fragment from Enterobacter agglomerans expressed MPS activity in E. coli JM109 without any noticeable alteration in pH of the medium. These results indicate that, though the production of organic acid by the microorganisms is one of the major factors but not the sole factor responsible for phosphate solubilization by bacteria. P is abundant in several soils and is one of the major nutrients limiting the plant growth. The overall P use efficiency following phosphate fertilizer application is low because of the formation of insoluble complexes [Vassilev & Vassileva, 2003]. Hence, frequent application of soluble forms of inorganic P is necessary for crop production and which leaches to the ground water and results in eutrophication of aquatic systems [Del Campillo et al., 1999]. In view of environmental concerns and current developments in sustainability, research efforts are concentrated on elaboration of techniques that involve the use of less expensive, though less bio-available sources of plant
nutrients such as rock phosphate and by application of PSB the agronomic effectiveness can be enhanced [Whitelaw, 2000].

IAA, a member of the group of phytohormones, is generally considered as a most important native auxin. All the selected PGPR isolates showed the production of IAA and were analysed qualitatively and quantitatively. Study revealed that the *Bacillus*, *Pseudomonas* and *Kebsiella* sp. are potent producer of IAA as compared with the *Cronobacter* isolate. IAA production was carried out at various temperature, pH, incubation time and in the presence of varying concentration of tryptophane. All the isolates have produced IAA in the range of 19-26 g/ml when NBRIP medium amended with 25 g/ml of tryptophane and under the absence of tryptophane isolates produced IAA in range of 15-20 g/ml of IAA indicates the increased efficiency of IAA production in presence of tryptophan. Similar results were reported by Kloepper et al. (1989).

It has been also reported that IAA production may vary depended on the species of bacteria and culture as well as physiological conditions. Moreover the isolates from the rhizosphere have more ability to produce IAA as compared to bulk soil. When medium supplemented with precursor tryptophane IAA production increased in the amount of 4, 5, 5, 6 g/ml for the isolates T-2, T-4, BR-1 and RR-1 respectively.

All the isolates which showed antifungal activity better were selected for the studies by dual culture method. The amounts and type of antifungal metabolites depend on the type of soils and nutrient availability [Duffy & Defago 1997]. It may be possible that some of these compounds at particular concentrations may be inhibitory to rhizobacteria. Plant pathogenic fungal strains *A. niger*, *Pn. roqueforti*, *A. flavus*, *A. solani*, *F. oxysporum*, *R. solani* were streaked along with PGPR strains *P. aeroginosa* T-2, *K. pneumoniae* T-4, *C. malonaticus* BR-1 and *B. subtilis* RR-1. All the isolates inhibited the progressive growth of test fungus. In order to show more antifungal index Isolate *P. aeruginosa* T-2 showed 75% inhibition index against *A. niger*, As the Isolate *K. pneumoniae* exhibited antifungal index 87% against *Pn. roqueforti*. Isolate *C.*
**malonaticus** BR-1 inhibited 80% growth of *P. roqueforti*. PGPR strain *B. subtilis* RR-1 showed 82% inhibition index against *A. solani*. *R. solani* was not promisingly inhibited by the PGPR strains as results showing inhibition index 11, 2, 2, 13 for the isolates T-2, T-4, BR-1 and RR-1 respectively. As all other plant pathogenic fungi inhibited by the isolates from the 65-87%.

All the four isolates produced GA₃ and quantity ranged from 15 to 27.8 µg/ml in N-broth and 11 to 23 µg/ml in NBRIP broth respectively. As it was seen during the study isolates produced more amount of GA₃ in the N-broth because of the availability of minor nutrients. The isolate *B. subtilis* RR-1 produced the maximum amount of GA₃ in both the NB and NBRIP 27.8 and 23 g/ml respectively, followed by T-2 (20 g/ml), T-4 (17 g/ml) and BR-1 (15 g/ml) in the NB while BR-1 (20 g/ml), T-2 (14 g/ml) and T-4 (11 g/ml) in NBRIP medium.

Siderophores are low molecular weight bio-molecules secreted by micro-organisms in response to iron starvation for acquisition of iron from insoluble forms by mineralization and sequestration [Lankford, 1973]. Although some siderophores are known to chelate other ions, their specificity and avidity for iron is the most consistent feature [Chincholkar *et al.*, 2007a]. Study showed production of siderophores by all rhizobacterial strains *P. aeruginosa* T-2, *K. pneumoniae* T-4, *C. malonaticus* BR-1 and *B. subtilis* RR-1.

The minimum amount of GA₃ was produced by *K. pneumoniae* T-4 (17 g/ml) in NBRIP and by *C. malonaticus* BR-1 (15 g/ml). The Bacillus isolates RR-1 produced the highest quantity of GB₃ in culture medium.

From the present study, we demonstrate that the natural subtropical soil supports a diverse group of potential PGPR isolates having multiple PGP traits. These P bacteria could serve as efficient biofertilizer candidates for improving the P-nutrition of crop plants. The advantage of using natural soil isolates over the genetically manipulated or the one which has been isolated from a different environmental set up is the easier adaptation and succession when inoculated into the plant rhizosphere. It is concluded from the present study that all the PSB
isolates produced multiple organic acids followed by a decrease in the pH of the culture medium thereby solubilizing the insoluble tricalcium phosphate. Use of these PSB as bioinoculants will increase the available P in soil, helps to minimize the P-fertilizer application, reduces environmental pollution and promotes sustainable agriculture.