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Isolation and characterization of plant growth promoting rhizobacteria associated with medicinal plant *Picrorhiza Kurroa*

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Abstract
Plant growth promoting rhizobacteria (PGPR) are naturally occurring soil bacteria that aggressively colonize plant roots and benefit plants by several mechanisms like IAA production, siderophore production, phosphate solubilization, etc. Phosphate solubilizing microorganisms play an important role in supplementing phosphorus to the plants by several mechanisms like lowering of pH by acid production and ion chelation and thus benefit plant growth and development. Therefore, the aim of this study was to determine the phosphate solubilizing potential of plant growth promoting rhizobacteria (PGPR) isolated from *Picrorhiza kurroa*. Forty bacterial isolates were selected (by modified replica plating technique) as the representative of the total plated population from the rhizosphere soil and rhizome/roots of the *Picrorhiza kurroa* from two locations of Chamba district. All the bacterial isolates were able to grow simultaneously on nutrient agar, Pikovskaya's, nitrogen free media and CAS media and selected for further screening for various plant growth promoting activities. Four phosphate solubilizing bacterial isolates exhibited very good production of chitinase enzyme with a zone size of 30-45mm.Maximum IAA production of 30.00µg/ml was exhibited by PkR(34)* and PkR(7b)*. The isolate Pk12(b) produced maximum per cent siderophore unit (27.21%). After screening, five isolates (PkR(7a)*,Pk7(B),Pk14(b), Pk12(d) and PC4) were selected for their ability to solubilize tricalcium phosphate(TCP). Out of the five isolates, isolate PkR(7a)* showed maximum P- solubilization of 320.00 mg/l at after 72 h of incubation at 35 ^{0}C in PVK broth. The isolate PkR(7a)* was identified to be *Bacillus subtilis* according to sequence analysis of 16S rDNA.

Keywords: *PGPR, phosphorus, IAA, Rhizobacteria*

1. Introduction
The medicinal plants are fast depleting due to over exploitation from their natural habitats, habitat destruction and unscrupulous collection. *Picrorhiza kurroa* Royle ex. Benth commonly known as "kutki" belongs to the family *Scrophulariaceae* is one such medicinal plant species which is facing a constant threat of extinction. Dried root and rhizome can be used for the purposes like hepatoprotection, immunomodulation, anti-asthmatic, anti-inflammatory antioxidant, anticholestic & laxative. To establish such plants in new environment the exploration of their microflora is must. In the present study this medicinal plant has been used for isolation of rhizobacteria and endorhizobacteria and ability of these rhizobacteria to perform various plant growth promoting activities. The soil micro-organisms constitute world's largest reservoir of biological diversity and are crucial to the functioning of terrestrial ecosystems. The rhizosphere, a narrow zone, adjacent to and influenced by, living plant roots (Kennedy, 1999) [1] is a site of high microbial activity in and around roots in soil (Sorensen, 1997) [2].
Plant growth promoting rhizobacteria (PGPR) are naturally occurring soil bacteria that aggressively colonize plant roots and benefit plants by several mechanisms (Cleyet- Marcel *et al*., 2001 and Kloepper, 1994) [3, 4]. Plant growth promoting rhizobacteria (PGPR) belong to diverse genera of *Azospirillum, Alcaligenes, Arthrobacter, Enterobacter, Erwinia, Acinetobacter, Bacillus, Burkholderia, Flavobacterium, Pseudomonas, Rhizobium* and *Serratia.* All of them are able to exert beneficial effects on plant growth (Tilak *et al*., 2005) [5].
Phosphorus is a major growth-limiting nutrient, and unlike the case for nitrogen, there is no large atmospheric source that can be made biologically available (Ezawa *et al.,* 2002) [6]. Although microbial inoculants are in use for improving soil fertility during the last century, however, a meager work has been reported on P solubilization compared to nitrogen fixation. Large amount of Phosphorus applied as fertilizer enters in to the immobile pools through precipitation reaction with highly reactive Al^{3+} and Fe^{3+} in acidic, and Ca^{2+} in calcareous or normal soils (Gyaneshwar *et al.,* 2002 and Hao *et al.,* 2002) [7, 8]. Efficiency of

P fertilizer throughout the world is around 10 - 25% (Isherword, 1998) [9], and concentration of bioavailable P in soil is very low reaching the level of 1.0 mg kg^{-1} soil (Goldstein, 1994) [10]. Soil microorganisms play a key role in soil P dynamics and subsequent availability of phosphate to plants (Richardson, 2001) [11]. Inorganic forms of P are solubilized by a group of heterotrophic microorganisms excreting organic acids that dissolve phosphatic minerals and/or chelate cationic partners of the P ions i.e. PO_4^{3-} directly, releasing P into solution (He *et al.,* 2002) [12]. Phosphate solubilizing bacteria (PSB) are being used as biofertilizer since 1950s (Kudashev, 1956 and Krasilinikov, 1957) [13, 14]. Release of P by PSB from insoluble and fixed / adsorbed forms is an important aspect regarding P availability in soils. There are strong evidences that soil bacteria are capable of transforming soil P to the forms available to plant. Microbial biomass assimilates soluble P, and prevents it from adsorption or fixation (Khan and Joergesen, 2009) [15]. Microbial community influences soil fertility through soil processes viz. decomposition, mineralization, and storage / release of nutrients. Microorganisms enhance the P availability to plants by mineralizing organic P in soil and by solubilizing precipitated phosphates (Chen *et al.,* 2006; Kang *et al.,* 2002 and Pradhan and Sukla, 2005) [16-18]. Thus the use of soil microorganisms which can fix atmospheric nitrogen, solubilize phosphorus or stimulate plant growth through synthesis of growth promoting substances and antagonize plant pathogenic fungi has gained importance over the use of chemical fertilizers and fungicides because of deleterious effect of the latter on the soil, plant health and ultimately on the living organisms (Glick *et al*., 1995) [19]. Although P content in soil on an average is 0.05%, only 0.1% of the total P present in soil is available to the plants because of its chemical fixation and low solubility. Under such conditions, microorganisms offer a biological rescue system capable of solubilizing the insoluble inorganic P of soil and make it available to the plants (Tilak *et al*., 2005) [5]. The analysis of culture filtrates has shown the presence of number of organic acids including citric, glycollic, fumaric, malic, oxalic, succinic, tartaric, malonic, lactic, 2-ketogluconic, gluconic, glyoxalic and α-ketobutyric acids (Gaur, 1990 and Illmer and Schinner, 1992) [20, 21].

In this work isolation of forty rhizospheric bacteria as well as endorhizobacteria was done and the isolates were examined for phosphate solubilization(both solid and liquid assay), siderophore production(both solid and liquid assay), IAA production, HCN production and Chitinase activity and finally the best isolate was identified by 16S rDNA analysis.

Material and Methods

Isolation and enumeration of rhizobacteria and endorhizobacteria by modified replica plating technique.

The *Picrorhiza kurroa* plants were carefully uprooted and upto one gram of rhizosphere soil and rhizome or root samples were used. The serially diluted suspension of soil / (rhizome and roots) was spread on pre-poured nutrient agar medium. After incubation of 24-48 h, the isolated colonies that developed on the nutrient agar medium (master plate) were replica plated onto the selective media: Nitrogen free medium for nitrogen fixing ability, CAS medium (Schwyn and Neilands, 1987) [22] for siderophore producing ability and Pikovskaya medium (Pikovskaya, 1948) [23], for phosphate solubilizing ability. All the colonies were transferred to the same position as the master plate with the help of wooden block, covered with sterilized velvet cloth. At the end of the incubation period, the location of the colonies appeared on the replica plates were compared to the master plate. The population of PSB was determined on the basis of ratio of number of P-solubilizers to total number of colonies on PVK medium and expressed as percentage.

Isolation of rhizobacteria

Soil samples along with rhizome and roots (upto 15 cms. depth) were collected from rhizosphere at Holi and Bharmour in district Chamba of Himachal Pradesh were shaken vigorously to remove the tightly adhered soil. One gram of this rhizosphere soil was placed in 9 ml of sterilized distilled water under aseptic conditions. The soil suspension was diluted in 10 fold series and the bacterial count was determined by the standard pour plate technique. Populations were expressed as colony forming unit (cfu) per gram of dry soil weight.

Isolation of endorhizobacteria

The rhizome and root sample was surface sterilized by 0.2 per cent mercuric chloride ($HgCl_2$) for three minutes followed by washing in sterilized distilled water. The surface sterility of roots was cross checked by incubating the surface sterilized roots in sterilized nutrient broth overnight. One gram of surface sterilized root sample was placed in 9 ml of sterilized distilled water and was ground to produce slurry using pestle and mortar under aseptic conditions. The root suspension was diluted in 10 fold series and bacterial count was determined by standard pour plate technique. Populations were expressed as colony forming unit (cfu) per gram of the root weight. The isolates were maintained on specific medium for further studies.

Phosphorus solubilization

Qualitative estimation of Phosphate Solubilization (Plate assay method) (Pikovskaya, 1948) [23]

The ability of bacteria to solubilize phosphorus was tested by streaking it on the PVK agar plates containing known amount of tricalcium phosphate ($Ca_3(PO_4)_2$). The plates were incubated at 37^0C for 48h. Each treatment was replicated three times. Solubilization of phosphorus was observed by yellow coloured zones produced around the isolated bacterial colonies. Percent solubilization efficiency and phosphate solubilization index was calculated as:

$$SE (\%) = \frac{Z+C}{C} \times 100$$

Where,

SE = Solubilization efficiency

Z = Halozone diameter (mm)

C = Colony diameter (mm)

Tricalcium phosphate solubilization in liquid medium

Pikovskaya's medium was used for the solubilization of phosphate. Fifty ml of medium was dispensed in 250 ml of Erlenmeyer flask containing 0.5 per cent tri calcium phosphate (TCP) and autoclaved at 15 psi for 20 min. The flasks were inoculated with 10% per cent of the bacterial suspension (OD 1.0 at 540 nm) and incubated at 35±2 ^0C under shake conditions for 72 h. Simultaneously, one control of PVK broth was run, containing TCP but no inoculum. Flasks were withdrawn at 72 h and contents were centrifuged at 15000 rpm for 20 min at 4ºC. The culture supernatant was used for determination of the soluble phosphorus as described by Bray and Kartz (1945) [24].

Assay of phosphate estimation (Bray and Kartz, 1945) [24]
The procedure essentially consisted of estimating soluble phosphorus formed by the action of phosphate solubilizing bacteria on tri-calcium phosphate. The soluble phosphorus formed was estimated calorimetrically and the results were extrapolated by standard curve drawn using potassium di-hydrogen phosphate.

Siderophore production
Estimation of Siderophore by Chrome-azurol-S (CAS) plate assay (Schwyn and Neilands, 1987) [22]
Siderophore production was detected by CAS plate assay method. Sterilized blue agar was prepared by mixing CAS (60.5 mg/50ml distilled water) with 5ml iron solution (1mM $FeCl_3.6H_2O$) and 5ml 10mM HCl). This solution was slowly added to hexadecyltrimethyl ammonium bromide (HDTMA) (72.9 mg/40ml distilled water). Thus, 50 ml CAS dye was prepared and poured into 500 ml nutrient agar and the plates were prepared.Twenty-four hours old culture of the test bacteria was spotted on pre-poured blue coloured CAS agar plates. Plates were incubated for 72 h, at 37^0C. Formation of a bright zone with a yellowish (hydroxamate), pinkish (catecholate) and whitish (carboxylate) colour in the dark blue medium indicated the production of siderophore.

Quantitative estimation of Siderophore using Chrome-azurol-S (CAS) liquid assay method (Schwyn and Neilands, 1987) [22]
0.1 ml of cell free extract of supernatant was mixed with 0.5 ml Chrome-azurol-S (CAS) assay solution along with 10 µl of shuttle solution (0.2M 5-Sulfosalicyclic acid). It was kept at room temperature for ten minutes and absorbance was recorded at 630 nm The minimal medium was used as a blank and the reference (r) was prepared using exactly the same components except the cell free extract of culture supernatant. The siderophore units were calculated using formula:

$$\text{Percent Siderophore unit} = \frac{A_r - A_s}{A_s} \times 100$$

Where,
A_r is defined as absorbance at 630nm of reference.
A_s is the absorbance at 630nm of the test.

Quantitative estimation of Indole-3-acetic acid (IAA) (Gorden and Paleg, 1957)
For the production of auxins, bacterial cultures were grown in Luria Bertani broth (amended with 5 mM L-tryptophan, 0.065 per cent sodium dodecyl sulphate and 1% glycerol) for 72 h at 37^0C under shake conditions. Supernatant was prepared/ collected by centrifugation of cultures at 15,000 rpm for 20 minutes and was stored at 4^0C.In measuring the IAA equivalents, 3 ml of supernatant was pipetted into test tube and 2 ml of Salkowski's reagent (2 ml of 0.5 M $FeCl_3$ + 98 ml 35% $HClO_4$) was added to it. The tubes containing the mixture left for 30 minutes (in dark) for the development of pink colour. Intensity of the colour was measured at 535 nm. Concentration of Indole-3-acetic acid was estimated by preparing calibration curve using Indole-3-acetic Acid (IAA, Hi-media) as standard (10-100µg/ml).

HCN production (Baker and Schippers, 1987) [26]
The bacterial cultures were streaked on prepoured plates of King's medium B amended with 4.4g/L glycine. Whatman No.1 filter paper strips were soaked in 0.5% picric acid in 0.2% sodium carbonate and was placed in between the petriplates. Petriplates were sealed with parafilm and were incubated at 37 ^{0}C for 1-4 days. Un-inoculated control was kept for comparison. Plates were observed for change of colour of filter paper from yellow to orange brown to dark brown.

Chitinase assay (Robert and Selitrennikoff 1988) [7]
The bacterial cultures were spotted on to the prepared minimal agar medium amended with 0.3% colloidal chitin and the plates were incubated at 30 ^{0}C for 7 days. Development of halo zone around the colony after addition of iodine was considered as positive for chitinase enzyme production.

Molecular characterization of selected isolate by 16S rDNA sequencing
Primer Designing
Primers were designed for the amplification of 16S rDNA from bacterial isolate. For that, GeneBank available sequences of 16S rDNA reported from different parts of the world were downloaded from the NCBI data base (web site: http://www.ncbi.nlm.nih.gov). These sequences were then aligned with either MULTIALIN program (web site: http://prodes.toulouse.inra.fr/multialin/multialin.html) based on algorithm as reported by Corpet (1988) [28] or by CLUSTALW program (web site: http://www.ebi.ac.uk/clustalw) based on the algorithm as described by Higgins *et al.* (1994) [29]. Based on the conserved regions in the aligned sequences the primers (Bf 5'GCAAGTCGAGCGGACAGATGGGAGC3' and Br 5'AACTCTCGTGGTGTGACGGGCGGTG 3') were designed keeping in mind the average GC content and the annealing temperature of the primer pairs. The designed primers were synthesized from Sigma.

PCR amplification of 16S rDNA
PCR reaction was carried out in 20 µl reaction containing ~50ng of template DNA, 20 pmoles of each primers, 0.2 mM dNTPs and 1 U Taq polymerase (M P Biomedical) in 1x PCR buffer. Reaction were cycled 35 times as 94ºC for 30 s, 58ºC for 30 s, 72 ºC for 1 min 30 s followed by final extension at 72ºC for 10 min. The PCR products were analyzed on 1% agarose gel in 1x TAE buffer, run at 100V for 1 hr. Gels were stained with ethidium bromide and photographed. Amplified PCR products were eluted from the gel using gel extraction kit (Hi Yield Gel/ PCR DNA Extraction Kit from Real Genomics).

Sequence analysis
Sequencing was done using primers with Big Dye terminator cycle sequencing kit according to the protocol of manufacturer (Xcleris lab). The sequence was aligned with corresponding sequences of 16s rDNA from the database using BLAST from the website http://www.ncbi.nlm.nih.gov/blast (Altschul *et al.*, 1997) [30]. Multiple alignments were generated by the MULTALIN program from the web site: http://prodes.toulouse.inra.fr/multialin/multialin.html (Corpet, 1988) [28]. Phylogenetic tree was constructed with the help of ClustalW from the website http://www2.ebi.ac.uk/clustalw/ (Higgins *et al.*, 1994) [29] and from the website www.ddbj.nig.ac.jp. Tree was viewed with the help of TreeView from the website http://taxonomy.zoology.gla.ac.uk/rod/treeview.html (Page, 1996).

Statistical Analysis
All the experiments were conducted in triplicates along with equal number of appropriate controls. Appropriate statistical/ mathematical tools will be utilized as per the requirements of the data. For screening and comparison of various treatments/ PGPR's and pathogens etc. the data will be subjected to analysis of variance technique using completely randomized design (CRD) developed by Gomez and Gomez (1976) [32].

Results
Bacterial isolation
Enumeration of culturable rhizospheric bacterial population associated with *Picrorhiza kurroa* using modified replica plating technique

Rhizosphere soil Samples	Rhizosphere soil bacterial population (×10^6 cfu / g soil)				Number of colonies forming halozone on PVK media	Per cent P solubilizers (%)*
	Nutrient agar medium (Master plate)	Nitrogen free glucose medium	Pikovskaya's (PVK) medium	Chome-azural-s (CAS Medium)		
1	71.60	30.30	47.30	5.00	40.00	84.56(9.19)
2	79.30	42.60	50.60	12.30	39.00	77.07(8.77)
3	43.30	24.60	46.00	6.00	43.30	94.13(8.07)
4	45.30	35.00	35.30	14.00	31.30	88.66(9.41)
5	49.60	26.60	35.60	13.00	26.30	73.87(8.59)
6	46.60	19.30	45.60	12.00	35.00	76.75(8.76)
7	43.30	34.30	33.30	11.00	25.00	75.07(8.66)
8	54.30	25.00	41.30	13.30	30.00	72.63(8.52)
9	72.30	41.30	49.30	13.30	39.00	79.10(8.89)
10	72.30	33.00	44.00	14.30	32.00	72.72(8.52)
11	43.60	17.60	23.30	16.00	21.00	90.12(9.49)
12	71.00	10.60	34.00	5.00	32.30	95.00(9.74)
13	79.30	22.60	35.00	3.00	29.00	82.85(9.10)
14	45.00	12.30	19.30	5.60	15.00	77.72(8.81)
CV%	23.98	37.36	25.09	45.91	25.15	8.80

Figures in parenthesis are square root transformed values

$$* \frac{\text{Number of colonies forming halozone on PVK media}}{\text{Total number of colonies on PVK media}} \times 100$$

A summary of population level of bacteria colonizing *Picrorhiza kurroa* rhizosphere of randomly selected samples from two locations of Holi and Bharmour is presented in the above Table. The colony forming units (cfu), determined for rhizosphere soil were rather different for different samples and varied with the medium used for enumeration. Among the fourteen samples, sample 2 and sample 13 had the highest number (79.3×10^6 cfu/g soil) whereas sample 3 and sample 7 had the lowest number (43.3×10^6 cfu/g soil) on the nutrient agar medium. The minimum coefficient of variation (23.98%) was observed for rhizospheric soil population on nutrient agar. Enumeration on the other thee differentiated and selective media was done by modified replica plating technique using master plate (nutrient agar) (Plate 1). After replica plating, sample 2 had the highest number (42.6×10^6 cfu/g soil) and sample 12 had the lowest number (10.6×10^6 cfu/g soil) on nitrogen free glucose medium. Similarly sample 2 accounted for the highest rhizospheric soil population (50.6×10^6 cfu/g soil) on PVK medium, however, sample 14 had the lowest population (19.3×10^6 cfu/g soil). On CAS medium the highest rhizospheric soil population was obtained in sample 11 (16.0×10^6 cfu/g soil) and minimum was obtained in case of sample 13 (3.0×10^6 cfu/g soil). Statistical analysis of rhizospheric soil population revealed that minimum coefficient of variation (25.10%) was observed on PVK medium. The per cent phosphate solubilizer of the total rhizosphere population on PVK medium for different samples, ranged between minimum (72.63%) in sample 8, to maximum (95.00%) in sample 12, respectively.

Endophytic bacterial population by surface sterilization and dilution technique
The experiment was conducted in order to determine whether roots of *Picrorhiza kurroa* are inhabited with bacteria and also to develop a protocol for isolation of endophytic bacteria. Endophytic bacteria were isolated from internal plant tissues using surface sterilization – trituration – plating technique.
The results represented in the above table, revealed that roots of all the plants collected from both the locations harboured bacteria capable of growth on the different media used. The maximum endorhizobacterial population on nutrient agar was obtained for root sample 5 (89.00×10^4 cfu/g root) and minimum endorhizobacterial population in case of in sample 2 (45.00×10^4 cfu/g root). The minimum coefficient of variation (22.60%) was observed for endophytic soil population on nutrient agar medium. Enumeration on the other thee media was done by modified replica plating technique using master plate (nutrient agar medium). After replica plating, in nitrogen free glucose medium the highest endorhizobacterial population (37.00×10^4 cfu/g root) was observed for sample 5. However, minimum endorhizobacterial population was observed in sample 2 (21.00×10^4 cfu/g root). Replica plating on PVK and CAS medium resulted in maximum endorhizobacterial population in case of sample 4 (50.00×10^4 cfu/g root) in PVK medium and sample 5 (20.00×10^4 cfu/g root) in CAS medium. However,minimum population was observed in case of sample 3 (27.00×10^4 cfu/g root) and sample 2 (7.00×10^4 cfu/g) on PVK and CAS media, respectively. Statistical analysis of endophytic soil population revealed that minimum coefficient of variation (24.41%) was observed on PVK medium.

Enumeration of culturable plant growth promoting endophytic bacterial population associated with rhizome and roots of *Picrorhiza kurroa* using modified replica plating technique

Rhizome/ Root Samples	Endophytic bacterial population ($\times 10^4$ cfu / g root)				Number of colonies forming halozone on PVK media	Per cent P solubilizers (%)*
	Nutrient agar medium (Master plate)	Nitrogen free glucose medium	Pikovskaya's (PVK) medium	Chome-azural-s (CAS medium)		
1	70	28	32	15	28	87.50(9.35)
2	45	21	35	7	25	71.40(8.45)
3	68	25	27	12	20	74.07(8.60)
4	84	32	50	17	40	80.00(8.94)
5	89	37	45	20	37	82.20(9.06)
CV%	22.60	24.50	24.41	35.93	25.14	3.83

Figures in parenthesis are square root transformed values

$$* \frac{\text{Number of colonies forming halozone on PVK media}}{\text{Total number of colonies on PVK media}} \times 100$$

The per cent phosphate solubilizer of the total endophytic bacterial population on PVK medium for different samples, ranged between minimum (71.40%) in sample 2 and maximum (87.50%) in sample 1.

Selection of Colonies Representative of Total Plated Population

After replica plating, the most predominant isolated colonies those simultaneously grow on all the four (Nutrient agar, Pikovskaya's, Nitrogen free glucose and CAS) media and were able to form a clear halo zone on the PVK agar medium, were isolated after comparison with the master plate. In this manner, 40 isolates were eventually selected from the master plates (nutrient agar). The morphologies of selected bacterial isolates associated with *Picrorhiza kurroa* collected from two different locations were identified. The majority of twenty eight isolates (70%) were from rhizosphere soil and twelve (30%) isolates were from endorhizosphere (isolated from internal root tissues). All the representative isolates were gram positive rods and variable colony morphology. These forty phosphate solubilizing bacterial isolates were selected for further screening.

Since the aim of present work was to find out phosphate solubilisers, hereafter only forty P solubilisers were screened

Colony morphology and gram's reaction of the selected phosphate solubilizing bacteria isolated from *picrorhiza kurroa*

Isolates	Colony morphology	Gram's reaction	Cell shape
Pk3A	Irregular, flat, lobate, creamish	+	rods
Pk4a	Irregular, flat, lobate, creamish	+	rods
PkH(4)	Irregular, flat, lobate, creamish	+	rods
PkH(5)	Irregular, flat, lobate, whitish	+	rods
Pk6(B)	Irregular, raised, lobate, creamish	+	rods
Pk6(c)	Circular, convex, entire, creamish	+	rods
Pk7(B)	Irregular, flat, lobate, creamish	+	rods
Pk8(A)	Circular, flat, entire, creamish	+	rods
Pk8(e)	circular, convex, entire, yellowish	+	rods
Pk9(B)	Circular, convex, entire, yellowish	+	rods
Pk11(c)	Irregular, flat, undulate whitish	+	rods
Pk12(a)	Irregular, umbonate, curled, creamish	+	rods
Pk12(b)	Circular, convex, entire, creamish	+	rods
Pk12(c)	Irregular, raised, lobate, creamish	+	rods
Pk12(d)	Circular, convex, entire, creamish	+	rods
Pk13(a)	Irregular, flat, lobate, creamish	+	rods
Pk13(b)	Irregular, flat, lobate, creamish	+	rods
Pk13(c)	Circular, convex, entire, creamish	+	rods
Pk14(a)	Circular, convex, entire, creamish	+	rods
Pk14(b)	Circular, convex, entire, creamish	+	rods
Pk14(c)	Circular, convex, entire, creamish	+	rods
PkR(2)*	Circular, convex, entire, yellowish	+	rods
PkR(21)*	Irregular, raised, lobate, creamish	+	rods
Pk(22)*	Irregular, raised, lobate, whitish	+	rods
PkR(32)*	Circular, raised, entire, creamish	+	rods
PkR(33)*	Circular, convex, entire, whitish	+	rods
PkR(34)*	Circular, convex, entire, yellowish	+	rods
PkR(5a)*	Circular, raised, entire, creamish	+	rods
PkR(5e)*	Irregular, umbonate, curled, creamish	+	rods
PkR(6a)*	Irregular, raised,undulate, creamish	+	rods
PkR(7a)*	Irregular, flat, lobate, creamish	+	rods
PkR(7b)*	Irregular, flat, undulate, whitish	+	rods
PkR(7c)*	Irregular, flat, undulate, whitish	+	rods
PC2	Irregular, flat, undulate, yellowish	+	rods
PC3	Irregular, flat, undulate, whitish	+	rods
PC4	Irregular, raised, erose, whitish	+	rods
PC7	Irregular, flat, erose, yellowish	+	rods
PC8	Irregular, flat, undulate, yellowish	+	rods
PC9	Irregular, flat, erose, yellowish	+	rods
PC13	Irregular, flat, erose, yellowish	+	rods

***:** Endorhizobacteria

Screening of phosphate solubilizing bacterial isolates for Chitinase and HCN production

The chitinase activity of all the forty isolates which is expressed as zone of clearance (mm) was found of varying ranges. The chitinase activity was found in 11 isolates (91.66%) out of selected 12 endophytes and only in 21 rhizosphere soil (75%) isolates out of total 28 isolates selected. The highest chitinase activity was observed in case of isolates Pk4a, PkR(6a)*,PkR(7a)*and PkR(7b)* with a zone size ranging between (30-45mm).The isolates Pk3A, PkH(4), PkH(5), Pk6(B), Pk7(B),Pk11(c), Pk12(a), Pk12(c), Pk12(d), PkR(22)*, PkR(32)*, PkR(34)*, PkR(5a)*, PkR(5e)*, PkR(7c), PC2, PC7 and PC8 had zone size ranging between 15-30mm and the isolates Pk6(c), Pk8(a), Pk12(b), Pk13(a), Pk13(b), Pk13(c),Pk14(c), PkR(2)*, PkR(33)* and PC13 were having zone size ranging between 0-15mm.However,the isolates Pk8(e), Pk9(B), Pk14(a), Pk14(b), PkR(21)*, PC3, PC4 and PC9 had no chitinase activity. The isolates also showed great variation for HCN production. Thee isolates (7.5%) viz., Pk14 (a), Pk14(c) and PC7 had very high HCN production in which the colour of entire filter paper got changed from yellow to brown. Seven isolates (17.5%) (Pk3A, Pk11(c), Pk12 (a), Pk14 (b), PkR(2)*, PC4 and PC8)showed change in colour of half of the filter paper from yellow to brown. Only the edges of the filter paper turned brown in case of 17 isolates (42.5%)(Pk4a, PkH(4), PkH(5), Pk7(B), Pk9(B), Pk12(b), Pk12(c), Pk12(d), Pk13(a), PkR(21)*, PkR(33)*, PkR(34)*, PkR(5e)*, PkR(6a)*, PkR(7b)*, PkR(7c) and PC2). However,13(32.5%) isolates Pk13(b), PkR(5a)*,Pk13(c), PkR(7a)*, PC3, PC9, PC13, Pk6(B), Pk8(A), Pk6(c), Pk8(e), PkR(32)* and PkR(22)*did not show HCN production activity.Overall,27 isolates (67.5%) had the ability to produce HCN out of which 8 isolates (20%) were endophytes.

Isolates	Chitinase activity**	HCN*** production
Pk3A	++	++
Pk4a	+++	+
PkH(4)	++	+
PkH(5)	++	+
Pk6(B)	++	-
Pk6(c)	+	-
Pk7(B)	++	+
Pk8(A)	+	-
Pk8(e)	-	-
Pk9(B)	-	+
Pk11(c)	++	++
Pk12(a)	++	++
Pk12(b)	+	+
Pk12(c)	++	+
Pk12(d)	++	+
Pk13(a)	+	+
Pk13(b)	+	-
Pk13(c)	+	-
Pk14(a)	-	+++
Pk14(b)	-	++
Pk14 (c)	+	+++
PkR (2)*	+	++
PkR (21)*	-	+
PkR (22)*	++	-
PkR (32)*	++	-
PkR (33)*	+	+
PkR (34)*	++	+
PkR (5a)*	++	-
PkR (5e)*	++	+
PkR (6a)*	+++	+
PkR (7a)*	+++	-
PkR (7b)*	+++	+
PkR (7c)*	++	+
PC2	++	+
PC3	-	-
PC4	-	++
PC7	++	+++
PC8	++	++
PC9	-	-
PC13	+	-

***:** Endorhizobacteria

**-: no zone +:zone size 0-15mm, ++:zone size 15-30mm and +++:zone size 30-45mm

*** - : No colour change of the filter paper from yellow to brown; + : change in colour on the edge of the filter paper; ++ : change in colour only half of the filter paper; +++ : change in colour of the complete filter paper from yellow to orange brown

Screening Of The Phosphate Solubilizing Bacterial Isolates For Multifarious Plant Growth Promoting Activities

A total of forty representative phosphate solubilizing bacterial isolates were screened for their multifarious plant growth promoting activities. The following Table revealed that selected bacterial isolates showed large variation in their production of indole-3-acetic acid (IAA).The isolates

PkR(34)* and PkR(7b)* produced 30µg/ml of IAA, while in isolates PkR(32)* and PC2 no IAA production was observed. The isolate PC9 produced 28 µg/ml of IAA and was statistically at par with the amount of IAA produced by isolates PkR(34)* and PkR(7b)*. The majority of the selected P-solubilizing isolates (95%) produced IAA to various extents.

All the forty isolates were evaluated for Phosphate solubilization (both quantitative and qualitative), Siderophore production (both quantitative and qualitative) and IAA production and the results were as follows

Isolates	Phosphate solubilization		Siderophore production		Indole -3- acetic Acid (µg/ml) production
	Phosphate solubilization index (PSI) **	Quatitative P-estimation (mg/l)●	Qualitative Siderophore estimation (Zone size, mm)	Quantitative estimation (%siderophore unit)○	
Pk3A	2.45	105.00	12.30	16.66(4.08)	13.00
Pk4a	2.27	135.00	7.30	14.96(3.86)	19.00
PkH(4)	2.56	115.00	13.00	15.57(3.94)	21.00
PkH(5)	2.61	130.00	5.00	12.05(3.46)	5.00
Pk6(B)	2.88	120.00	6.00	9.70(3.10)	14.00
Pk6(c)	2.50	100.00	8.00	14.28(3.77)	6.00
Pk7(B)	2.58	120.00	20.00	21.76(4.66)	14.00
Pk8(A)	2.35	135.00	4.00	4.08(2.00)	12.00
Pk8(e)	2.43	95.00	3.00	0.75(0.866)	3.00
Pk9(B)	2.50	95.00	5.00	21.30 (4.61)	6.00
Pk11(c)	3.00	125.00	17.60	16.41(4.04)	21.00
Pk12(a)	2.50	75.00	14.60	21.30(4.61)	10.00
Pk12(b)	2.80	50.00	10.00	27.21(5.21)	14.00
Pk12(c)	2.43	85.00	20.00	25.07(5.00)	13.00
Pk12(d)	2.38	100.00	19.00	16.16(4.01)	22.00
Pk13(a)	3.16	95.00	17.30	17.45(4.17)	6.00
Pk13(b)	2.80	80.00	11.00	10.42(3.21)	11.00
Pk13(c)	2.56	120.00	4.00	8.20(2.86)	3.00
Pk14(a)	3.00	90.00	8.00	15.38(3.92)	16.00
Pk14(b)	4.05	205.00	11.00	8.20(2.86)	21.00
Pk14(c)	3.42	40.00	5.00	10.42(3.22)	4.00
PkR(2)*	2.36	80.00	12.00	16.09(4.01)	9.00
PkR(21)*	2.40	100.00	8.30	14.61(3.820	5.00
PkR(22)*	2.32	55.00	7.60	13.61 (3.68)	8.00
PkR(32)*	2.54	110.00	19.60	17.31(4.15)	0.00
PkR(33)*	2.33	55.00	24.00	16.54(4.06)	16.00
PkR(34)*	2.35	70.00	14.30	8.64(2.93)	30.00
PkR(5a)*	2.78	90.00	11.30	12.38(3.50)	4.00
PkR(5e)*	2.62	90.00	11.60	16.00(3.99)	4.00
PkR(6a)*	2.65	100.00	19.60	17.03(4.12)	22.00
PkR(7a)*	2.73	320.00	12.00	0.75(0.86)	21.00
PkR(7b)*	2.82	20.00	5.00	2.04(1.42)	30.00
PkR(7c)*	3.30	90.00	7.00	11.31(3.35)	11.00
PC2	3.00	80.00	16.60	16.16(4.01)	0.00
PC3	2.75	90.00	10.30	6.00(2.42)	21.00
PC4	2.60	180.00	11.00	9.70(3.11)	18.00
PC7	3.07	75.00	21.00	18.64(4.30)	24.00
PC8	2.66	80.00	18.00	16.09(4.00)	11.00
PC9	3.05	50.00	21.00	17.70(4.20)	28.00
PC13	3.42	60.00	18.30	12.03(3.46)	16.00
$CD_{0.05}$	1.05	4.05	3.08	0.36	2.55

(r= 0.09) (r= 0.10)

* **:** Endorhizobacteria

**: $\frac{\text{Clear zone diameter (colony + halo zone)}}{\text{Growth diameter}}$;

●**T**-C; Where, T= Inoculated PVK with TCP, C (uninoculated PVK with TCP)

○:% Siderophore unit= $\frac{Ar - As}{As}$ X 100

A_r = Absorbance at 630nm of reference; A_s = Absorbance at 630 nm of test sample;

The phosphate solubilizing activity of the selected bacterial isolates were compared on the basis of their phosphate solubilizing index (PSI) in PVK agar Medium and P-solubilization (mg/l) in PVK broth medium. The results revealed that in PVK agar medium, Pk14(b) showed maximum phosphate solubilizing index (4.05) and minimum was recorded in Pk4a (2.27).The PSI of isolates Pk11(c) (3.0), Pk13(a) (3.16), Pk14(a) (3.0), Pk14(c) (3.42), PkR(7c)*(3.3), PC2 (3.0), PC7 (3.07), PC9 (3.05) and PC13 (3.42) was found statistically at par with PSI of isolate Pk14(b) (4.05).

In liquid PVK medium, maximum P- solubilization was recorded for endophytic isolate PkR(7a)* (320.0 mg/l) followed by Pk14(b) (205.0 mg/l) which was significantly lower than PkR(7a)*. The isolate Pk14(c) solubilized minimum TCP with release of 40.0 mg/l phosphorus.TCP

solubilization of all the isolates was significantly lower than TCP solubilization in PkR(7a)*(320.0 mg/l). The maximum PSI was not related to the maximum P solubilization in liquid medium. The correlation coefficient (r=0.09) between PSI on solid medium and P solubilization in liquid medium by the bacterial isolates was found to be positive and non-significant. The siderophore production of the selected bacterial isolates were compared on the basis of their zone size (mm) and per cent siderophore unit. All the isolates produced siderophore. The results revealed that endophyte PkR(33)* produced maximum zone size (24.0mm) which was statistically at par with 21.0 mm for PC7 and PC9 on Chome-azurol-S (CAS) solid medium and minimum zone size was observed in isolate Pk8(e) (3mm)

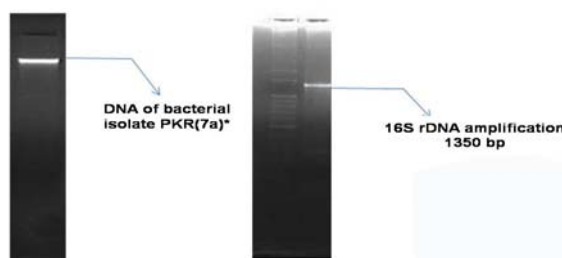
Quantitative estimation of siderophore using Chome-azurol-S (CAS) liquid assay revealed that Pk12 (b) produced maximum per cent siderophore unit (27.21%) which was statistically at par with 20.0 per cent siderophore unit for isolate PK12(c). Minimum per cent siderophore unit (0.75%) was found for isolates PkR (7a)* and Pk8(e). The correlation coefficient (r=0.10) between qualitative and quantitative siderophore estimation was found to be positive and non-significant.

Molecular characterization (16s rdna sequencing) of selected bacterial isolate

Since the aim of the present study was isolation of most efficient phosphate solubiliser so molecular identification of that very fungus was done

PCR amplification

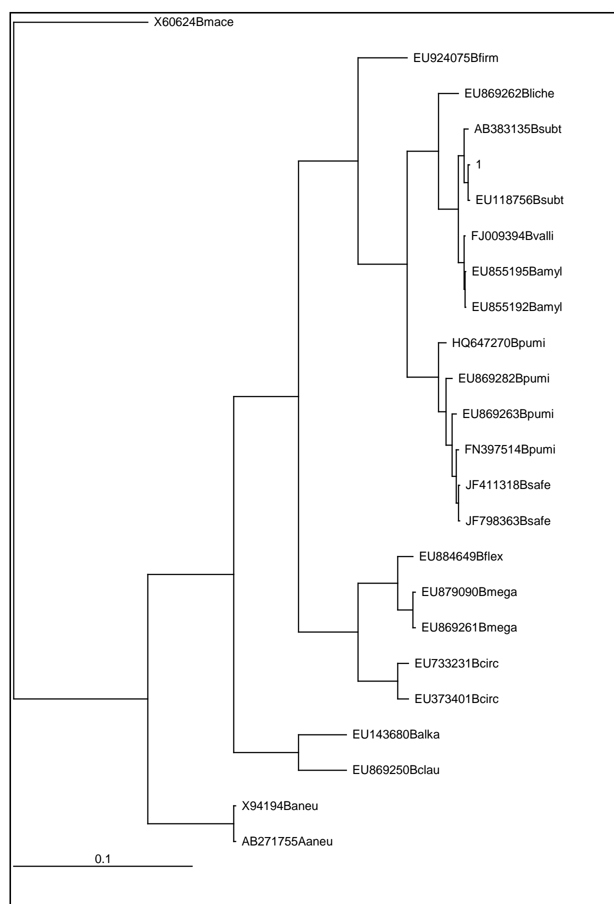
Designed primers were used successfully for amplification of 16S rDNA from Bacterial isolate (PkR(7a)*). Amplicon of expected size, i.e. ~1350 bp was obtained as in Figure. The PCR product was eluted from gel, and sequenced using PCR primers. Dendogram based on phylogenetic analysis presented in dendogram shows that *Bacillus* isolate (PkR(7a)*) is clustered with *Bacillus subtilis*. The sequence of respective isolate was submitted to NCBI under accession no. JN559852 and sequence of the isolate is as shown under.



Gel photograph showing amplicon of 16S rDNA of *Bacillus subtilis* (PkR(7a)*) Lane 1:100 bp ladder ;Lane 2:1350 bp amplification

PkR(7a)* complete (*Bacillus subtilis*)

AGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCG
GCGGACGGG
TGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGG
ATAACTCCGGGAAACCGGGGCTAATACCGGATGGTT
GTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTT
CGGCTACCACTTACAGATGGACCCGCGGCGCATTAG
CTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGAT
GCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGG
GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGC
AGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACG
GAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATC
GTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTT
CGAATAGGGCGGTACCTTGACGGTACCTAACCAGAA
AGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAAT
ACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCG
TAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTG
AAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAA
CTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAAT
TCCACGTGTAGCGGTGAAATGCGTAGAGATGT



UPGMA phylogenetic tree based on 16S rDNA sequence data of *Bacillus subtilis* (PkR(7a)*) and related strains

Phosphate solubilization and concomitant production of plant growth promoting activities under optimized conditions

Isolates	P solubilization$^{\alpha}$ (mg/l)	Final pH of supernatant	Viable count ($\times 10^6$ cfu/ml)	IAA Production** (µg/ml)	% siderophore*** unit	% growth inhibition against *Dematophora necatrix*•
PkR(7a)*	320.00	4.72	49.00	21.00	0.75	0.00
PC4	180.00	5.03	45.00	18.00	9.70	40.00
T value	82.61	2.58	2.36	1.77	74.31	33.38
					r = 0.99	

Optimized conditions:
* endorhizobacteria incubation period 72 hs Temperature 35 ^{0}C pH 7.0
Age of inoculum 24 h old Size of inoculum 10% TCP conc. 0.5%

α T- C; Where, T = Inoculated PVK with TCP, C (Uninoculated PVK with TCP)
** T-C; Where, T: IAA produced in inoculated,C: IAA produced in control

*****%** Siderophore unit = $\frac{A_r - A_s}{A_r}$ X 100
A_r = Absorbance at 630nm of reference; A_s = Absorbance at 630 nm of test sample

● I = $\frac{C-T}{C}$ × 100
Where, I = Per cent growth inhibition, C = Growth of fungus in control,T = Growth of fungus in treatment

Phosphate Solubilization And Concomitant Production Of Pgpr Activities Under Optimized Conditions

The isolates viz., PkR(7a)* and PC4 were compared for their average performance with respect to P solubilization, final pH of supernatant, viable count, IAA production, percent siderophore unit and percent growth inhibition against *Dematophora necatrix*. Though Fischer's t-test the computed value of t-statistic have been presented. The results revealed that isolates differ significantly for all characteristics except IAA production. Moreover, isolate PkR (7a)* is attaching significantly higher average value to P solubilization and viable count and PC4 is attaching significantly higher average value to final pH of supernatant,% siderophore unit and% growth inhibition against *Dematophora necatrix*. Moreover, the correlation coefficient between% siderophore unit and% growth inhibition against *Dematophora necatrix* (r=0.99) was positive and almost perfect which indicated that the two parameters were closely related.

Discussion

The samples for bacterial isolates of rhizosphere and endophytic bacteria capable of growth on different media were taken up by uprooting the medicinal plant *Picrorhiza kurroa* grown in its natural habitat. The results indicate that PSB colonized the *Picrorhiza kurroa* rhizosphere and also colonization of internal root tissues is observed. The presence of large population of bacterial isolates in all the samples may be due to positive influence exerted by root exudates in microbial colonization. Since root exudates are the primary source of nutrients for rhizosphere micro-organisms, rhizosphere competence implies that plant growth promoting rhizobacteria (PGPR) are well adapted to their utilization (Van and Van, 1995 and Lugtenberg *et al*., 1999) [33, 34]. Generally bacterial populations are larger in the rhizosphere soil than in the roots.

All the collected samples of rhizosphere soil and rhizome/roots were evaluated for P-solubilizing bacteria as well as for ability to grow on nitrogen free medium and production of siderophore. Rhizobacteria exhibiting all these three plant growth promoting traits were found in all the samples. The population count was higher in rhizosphere soil than those in roots. The occurance of higher population of P-solubilizers in rhizosphere soil is of direct significance to the plants growing in wild habitats as PSB's help in mobilization of insoluble P near the roots, especially in P-deficient soils. The positive rhizosphere effect of perennial plants on activities of microorganisms have been widely reported, though limited information is available about the rhizosphere influence on P-solubilizing, free living nitrogen fixing and siderophore producing microorganisms. The number of P-solubilizing microorganisms was invariably higher than those able to grow on nitrogen free medium and on CAS medium. The abundance and diversity of P solubilizers is different in different samples although total bacterial count on the PVK media for rhizosphere soil is very high. The rhizosphere soil showed variation among phosphate solubilizing bacteria from 19.30 × 10^6 cfu g^{-1} soil to 50.60 × 10^6 cfu g^{-1} soil and endophytic root PSB count was 27.00 × 10^4 cfu g^{-1} root to 50.00 × 10^4 cfu g^{-1} root which was found to be the similar with the study that PSB count showed large variation from 3-67 X 10^6 cfu g^{-1} and biodiversity within the crop and place of sampling (Kundu *et al*., 2009) [35]. The number of phosphate solubilizing bacterial isolates was identified by measuring radii of clear zone around their colony. Per cent phosphate solubilizing bacteria for rhizosphere population varied from 72.63% to 95.00% and for endophytic root tissues varied from 71.40% to 87.50%.

Rhizospheric bacteria showed higher percentage of PSB, thus twenty eight rhizospheric PSB isolates and twelve endophytic PSB isolates were isolated. The isolation was done by using modified replica plating technique on nitrogen free glucose medium, Pikovskaya's medium and Chrome-azural-s(CAS) medium from the master plate (nutrient agar). All the 40 representative phosphate solubilizing bacterial isolates were Gram positive rods and had variable colony morphology. The most predominant P-solubilizing rhizobacterial isolates from *Picrorhiza kurroa* growing in wild habitat in location under this study were Gram positive rods. The dominance of genus *Bacillus* as a P solubilizing bacteria in the rhizosphere of several crops has been reported earlier (Illmer and Schinner, 1992; Motsara *et al*., 1995 and Tilak and Reddy, 2006) [21, 36, 37]. The majority of P solubilizing bacteria associated with *Salix alba* from Lahaul and Spiti valleys of Himachal Pradesh were reported to be Gram positive rods (Chatli *et al*., 2008) [38].

However, acetylene reduction assay (ARA) (not conducted in the study) is an indicator of nitrogenase activity, thereby confirming the notion that many bacteria are excellent scavengers of nitrogen.

Further selection of all the representative bacterial isolates was based on the ability of the isolates to exhibit various plant growth promoting activities in tendem. Out of forty isolates, four isolates produced very large zone size (30-45mm) on chitinase media screening of the most efficient PSB *in vitro* was based on the ability of the isolate to release phosphorus into the culture medium and its relationship with the phosphate solubilizing index (PSI) based on colony diameter and halozone for each isolate. Since, in some cases, there have been contradictory results between plate halozone detection and P- solubilization in the liquid culture (Rodriguez and Fraga, 1999) [39]. In the present study, positive but non-significant correlation (r=0.09) was observed between qualitative and quantitative P- solubilization. This finding is in support to the moderate positive correlation between the TCP solubilizing efficiency on solid medium and amount of phosphate solubilized in liquid medium by pseudomonads (Dave and Patel, 1999) [40] and least correlation was found with P- solubilized and colony + halozone diameter (Alam *et al*., 2002 and Srivastav *et al*., 2004) [41, 42].

Our finding is in contrast to the pattern of phosphate solubilization by PSB in qualitative assay correlated well with the quantitative assay (Edi- Premono *et al*., 1996; Kumar and

Narula, 1999 and Mehta and Nautiyal, 2001) [43, 44, 45]. This is because most PSB show fluctuation in their behavior of production of halozone on solid medium but it is not necessary that PSB with larger halozone would solubilize more phosphorus. In the present study, a positive non-significant correlation (r=0.10) was observed between qualitative and quantitative siderophore estimation. The exhibition of multiple plant growth promoting traits by a single strain of PGPR has been reported earlier also (Hamdali *et al*., 2008) [46]. Bacterial plant growth promotion is a well-established and complex phenomenon, and is often achieved by the activities of more than one plant growth promoting traits exhibited by associated bacterium (Dastager *et al*., 2011) [47]

Out of forty phosphate solubilizing bacterial isolates, three isolates (Pk7(B), Pk14(b), and PC4) possess the capability to perform main plant growth promoting functions viz. solubilization of P, nitrogen fixation, IAA production, siderophore production The isolate PkR(7a)*, possessed very high ability to solubilize phosphate (320 mg/l).

Growth and tricalcium phosphate (tcp) solubilization in liquid medium

Different form of insoluble phosphates such as tricalcium phosphate (TCP), rock phosphate (RP), hydroxyapatite (HAP), dicalcium phosphate (DAP) have been used to determine the phosphate solubilizing activity of microorganisms in Pikovskaya's medium (Chabot *et al*., 1998 and Kundu *et al*., 2002) [48, 49]. Tricalcium phosphate has been reported to be readily solubilized than rock phosphate (Srivastav *et al*., 2004) [42] and in the present study, TCP was selected as substrate for determining the phosphate solubilizing potential of selected bacterial isolates.

Also an inverse correlation (r=-0.70) was observed between pH and amount of P released in liquid media which was in agreement with the findings of who showed a strong relationship between a drop in the pH and drastic increase in soluble phosphate concentration (r=-0.69).

The release of soluble phosphate from tricalcium phosphate usually involves the production of organic acids and a decrease in the pH of the medium (Carrillo *et al*., 2002 and Puente *et al*., 2004) [50, 51].

In the present study, maximum soluble phosphorus 320.00 mg/l with final pH of the supernatant 4.72 was obtained as a result of P- solubilizing activity of PkR(7a)* from 1000 mg/l of total phosphorus in the TCP added initially. All the selected bacterial isolates showed decrease in final pH of the supernatant with increase in P- soubilization of the medium. It has been suggested that microorganisms which decrease the medium pH during growth are efficient P- solubilizer (Nautiyal *et al*., 2000) [52]. In our study, maximum growth coincide with the maximum amount of P- solubilization and is in agreement with the earlier reports on P- solubilization (Promod and Dhevendaran, 1987; Chabot *et al*., 1998 and Vasquez *et al.,* 2000) [53, 48, 54]

P-solubilization and concomitant production of plant growth promoting activites

It is a well-established fact that improved phosphorus nutrition influences overall plant growth and root development (Jones and Darrah, 1994) [57]. Phosphate solubilizing micro-organisms benefit plant growth and development not only by the increase in uptake of phosphorus but are often combined with production of other metabolites, which take part in biological control against soil –borne plant pathogens. The present studies show the potential of P solubilizing PkR(7a)* and PC4 for the simultaneous production of indole acetic acid. The isolate PC4 also produced siderophore and had antifungal activity, however, neither siderophore production nor antifungal activity was observed in isolate PkR(7a)* which suggests the possible role of siderophores in antifungal activity. Siderophore production by the isolates assumes significance for iron nutrition of plants grown under iron deficient conditions. Other workers (Buysens *et al*., 1996; Kirner *et al*., 1998 and Srivastav *et al*., 2004) [58, 59, 42] have repored that the inhibition of growth of phytopathogens was due to the production of some specific siderophores, antibiotics, secondary metabolites or hydrolytic enzymes and HCN. Vassilev *et al*. 2006 [56] for the first time demonstrated the capacity of *B. thuringiensis* to solubilize insoluble inorganic phosphate and simultaneously produce IAA in a repeated batch fermentation process. HCN production by rhizospheric bacteria has been variably viewed, while it is considered effective from the biocontrol point of view. The capacity to produce phytoharmones like IAA, is a desirable characteristic of PGPR (Vessey, 2003) [55].

Phylogenetic analysis of selected bacterial isolate

Bacillus isolate showed maximum homology 99% with Chinese isolate (EU118756) of *Bacillus subtilis*, Vietnam isolate (AB383135) of *Bacillus subtilis*, Chinese isolate (FJ009394) of *Bacillus vallismortis* and minimum homology 77% with Indian isolate of *Bacillus firmus*. Phylogenetic analysis showed that Indian (PkR (7a)*) isolate is more closely related to Chinese isolate (EU118756) isolate of *Bacillus subtilis*.

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