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Isolation of phosphate solubilising rhizobacteria and endorhizobacteria from medicinal plant *Picrorhiza kurroa* and their optimization for tricalcium phosphate solubilization

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Abstract
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 show P- solubilization on Pikovskaya's, solid medium and were also grown on liquid Pikovskaya's medium for estimating their P-solubilizing potential. out of the forty isolates five isolates (PkR (7a)*,Pk7(B),Pk14(b), Pk12(d) and PC4) showed maximum P- solubilization, solubilizing 320.00 mg/l, 120.00 mg/l, 205.00 mg/l,100.00 mg/l and 180.00 mg/l respectively. These five isolates were selected for their ability to solubilize tricalcium phosphate(TCP). in four different media viz., Pikovskaya's Broth, Luria Bertani Broth, Nutrient Broth, National Botanical Research Institute Phosphate Growth Media and Pikovskaya's Broth was found to be the best medium for growth and P-solubilization. Further two isolates PkR (7a)*and and PC4 were selected for optimization of various parameters like effect of incubation period, temperature, pH, age of inoculum, size of inoculums, higher concentration of tricalcium phosphate on tricalcium phosphate solubilization and it was found that the isolate PkR (7a)* showed maximum P- solubilization of 320.00 mg/l at pH 7.0, 0.5 per cent TCP concentration, 10% of inoculum size and 24 h old culture after 72 h of incubation at 35 ^{0}C in PVK broth. This isolate was identified as *Bacillus subtilis* by 16S rDNA sequencing.

Keywords: Rhizobacteria, tricalcium phosphate, phosphate solubilization

Introduction
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) [1]. Root development, stalk and stem strength, flower and seed formation, crop maturity and production, N-fixation in legumes, crop quality, and resistance to plant diseases are the attributes associated with phosphorus nutrition. 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. Soil P 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 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) [2, 3]. Efficiency of P fertilizer throughout the world is around 10 - 25% (Isherword, 1998) [4], and concentration of bioavailable P in soil is very low reaching the level of 1.0 mg kg^{-1} soil (Goldstein, 1994) [5]. Soil microorganisms play a key role in soil P dynamics and subsequent availability of phosphate to plants (Richardson, 2001) [6]. 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) [7]. Phosphate solubilizing bacteria (PSB) are being used as biofertilizer since 1950s (Kudashev, 1956 and Krasilinikov, 1957) [8, 9]. 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) [10]. 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) [11, 12, 13].

Though much information is available on activity of soil micro-organisms and plant growth promotion for annual crops (Glick, 1995 and Bashan, 1998) [14, 15], very limited information is available in respect of PGPR associated with medicinal plants such as *Picrorhiza kurroa*. The aim of the present study was not only to isolate and estimate the phosphate solubilising potential of rhizobacteria and endorhizobacteria but also to optimize the parameters like media, incubation period, pH, temperature, age of inoculum, size of inoculums, higher concentration of tricalcium phosphate required for maximum solubilization of tricalcium phosphate

Material and methods

Isolation and enumeration of rhizobacteria and endorhizobacteria

The *Picrorhiza kurroa* plants were carefully uprooted and upto one gram of rhizosphere soil and rhizome and root samples were used. 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. 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) for siderophore producing ability and Pikovskaya medium (Pikovskaya, 1948), for phosphate solubilizing ability.

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

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 ^{0}C 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 (%) = $\frac{Z+C}{C} \times 100$

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 ^{0}C 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 4 ºC. The culture supernatant was used for determination of the soluble phosphorus as described by Bray and Kartz (1945). Estimation of soluble phosphorus formed by the action of phosphate solubilizing bacteria on tri-calcium phosphate was done calorimetrically and the results were extrapolated by standard curve drawn using potassium di-hydrogen phosphate.

An aliquot (0.1-1.0 ml) from the culture supernatant was made to final volume of 25 ml with distilled water and 5 ml ammonium molybdate was added. The mixture was thoroughly shaken. The contents of the flasks were diluted to 20 ml. Added 1.0 ml of chlorostannous acid was added and its volume was made to 25ml. in the volumetric flask. The contents were mixed thoroughly and the blue coloured intensity was measured after 10 minutes at 660 nm. An appropriate blank was kept in which all reagents were added except the culture.

P solubilization = T- C Where,

T = PVK with TCP, inoculated

C = PVK with TCP, un-inoculated

Molecular characterization of selected isolate by 16S rDNA sequencing

DNA isolation

Bacterial isolate (PkR (7a)*) was grown overnight at 37 ºC in nutrient broth at 200 rpm. The cells were harvested and processed for DNA isolation. Total bacterial DNA was isolated from 5 ml overnight culture using total DNA isolation kit (Real Genomic DNA Extraction Kit).

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) [18] or by CLUSTALW program (web site: http://www.ebi.ac.uk/clustalw) based on the algorithm as described by Higgins *et al.* (1994) [19]. 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 94 ºC for 30 s, 58 ºC for 30 s, 72 ºC for 1 min 30 s followed by final extension at 72 ºC 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). Multiple alignments were generated by the MULTALIN program from the web site: http://prodes.toulouse.inra.fr/multialin/multialin.html (Corpet, 1988) [18]. Phylogenetic tree was constructed with the help of ClustalW from the website http://www2.ebi.ac.uk/clustalw/ (Higgins *et al.*, 1994) [19] 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) [22].

Results

Population densities associated with rhizosphere soil of *Picrorhiza kurroa* 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 Table 1. 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). 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.

The experiment was conducted in order to determine whether roots of *Picrorhiza kurroa* are inhabited with bacteria The results (Table 2) 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. 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.

The results (Table 3) 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.

Out of the 40 isolates only 5 isolates showing maximum P-solubilization were selected and screened for further optimization. In order to determine the best medium for selected bacterial isolates PkR (7a)*, Pk14(b), Pk12(d), Pk7(B) and PC4 for TCP solubilization this experiment was conducted. The results (Table 5) revealed that the P-solubilization, viable count and final pH of supernatant varied greatly with different media. TCP solubilization was found maximum in PVK broth followed by NBRIP broth and TCP solubilization decreased considerably in Luria broth and nutrient broth. Comparison of different media(PVK,LB,NB and NBRIP) revealed that maximum mean P- solubilization (183.00mg/l) and maximum mean drop in final pH (4.98) of the supernatant was observed in PVK broth which was significantly higher than all other media irrespective of the bacterial isolates. However, interaction studies revealed that maximum P- solubilization (320.00mg/l) and maximum drop in final pH (4.72) of the supernatant and highest growth (63.0×10^6 cfu/ml) was observed for isolate PkR(7a)* in PVK broth which was significantly higher than P- solubilization and drop in final pH of the supernatant by the other bacterial isolates. A significant decrease in P- solubilization and

corresponding drop in final pH of the supernatant was observed by all the bacterial isolates in rest of the three media. Similar trend was observed in case of viable count and maximum mean viable count (54.80×10^6 cfu/ml) was found in PVK broth and minimum mean viable count (35.20×10^6 cfu/ml) was found in NB. The mean viable count in PVK broth was significantly higher than mean viable count in rest thee media. Also the correlation coefficient between pH and P-solubilization (r= -0.70) was significant and revealed that there was an inverse correlation between the two parameters.

In order to determine the optimum incubation period for selected bacterial isolates (PkR(7a)* and PC4) for TCP solubilization this experiment was conducted. The results (Table 6) revealed that the P-solubilization and viable count increased while pH decreased with increase in incubation period from 24 to 72 h. Comparison of mean P- solubilization and corresponding mean drop in pH for selected bacterial isolates irrespective of the incubation period revealed that maximum mean P- solubilization (204.00 mg/l) and maximum mean drop in final pH (5.21) of the supernatant was observed for isolate PkR(7a)* which was significantly higher than PC4.Comparison of different incubation periods (24, 48, 72 and 96 h) revealed that maximum mean P-solubilization (252.50 mg/l) and maximum mean drop in final pH (4.86) of the supernatant was observed after 72 h of incubation which was significantly higher than all other incubation periods irrespective of the bacterial isolates with a non-significant decrease in pH. However, interaction studies revealed that maximum P- solubilization (320.00 mg/l) and maximum drop in final pH (4.72) of the supernatant was observed for isolate PkR(7a)* at 72 h of incubation which was significantly higher than P- solubilization by other bacterial isolate used in the study with a non-significant drop in final pH of the supernatant. A significant decrease in P-solubilization with a corresponding non-significant drop in final pH of the supernatant was observed by the bacterial isolates with increase in incubation period from 72 to 96 h.The maximum mean viable count (43.00×10^6 cfu/ml) was observed at 72 h of incubation. A comparison of mean P-solubilization for selected bacterial isolates (PkR(7a)* and PC4) revealed that maximum mean P- solubilization (188.70mg/l) and maximum mean drop in final pH (5.28) of the supernatant was observed for isolate PkR(7a)* which was statistically higher than mean P- solubilization (111.25 mg/l) for PC4 irrespective of the incubation temperature. Also comparison of different temperatures (25^0, 30^0, 35^0 and 40 ^{0}C) revealed that maximum mean P- solubilization (242.50mg/l) and corresponding maximum mean drop in final pH (4.87) of the supernatant was observed at 35 ^{0}C which was significantly higher than the P- solubilization at other incubation temperatures used in the study irrespective of the bacterial isolates.However, interaction studies revealed that PkR(7a)* showed maximum P- solubilization (310.00 mg/l) with corresponding final pH (4.70) which was significantly higher than the P- solubilization by the other bacterial isolate used in the study at 35 ^{0}C.Further increase in temperature from 35^0 to 40 ^{0}C resulted in a significant decrease in P- solubilization for both bacterial isolates studied.Similar trend was observed in case of viable count by selected bacterial isolates. The maximum mean viable count was found to be (44.50×10^6 cfu/ml) at 35 ^{0}C which was significantly higher than mean viable count (22.00×10^6 cfu/ml) at 25 ^{0}C and mean viable count (31.00×10^6 cfu/ml) at 40 ^{0}C.Therefore, 35 ^{0}C was selected as the best incubation temperature for maximum P-solubilization and viable count by both the bacterial isolates.

Effect of size of inoculum on tricalcium phosphate solubilization by selected bacterial isolates revealed (Table 10) that the solubilization of tricalcium phosphate was significantly affected by varying concentration of inoculum (1%, 5%, 10% and 15%) at 35 ^{0}C after 72 h of incubation. Comparison of mean P- solubilization for selected bacterial isolates irrespective of the size of inoculum revealed that maximum mean P- solubilization (292.50 mg/l) and corresponding maximum drop in final pH (4.80) was observed for isolate PkR(7a)* which was significantly higher than P- solubilization (157.50mg/l) and mean drop in final pH (5.22) by PC4. Comparison of different concentrations of inoculums revealed that maximum mean P- solubilization (252.50 mg/l) and maximum mean drop in final pH (4.87) of the supernatant was observed at 10% inoculum size which was significantly higher than P- solubilization at other concentration of inoculum (1%, 5%, 10% and 15%) irrespective of the bacterial isolates.However, interaction studies revealed that maximum P- solubilization (320.0 mg/l) and maximum drop in final pH (4.73) of the supernatant was observed for isolate PkR(7a)* which was significantly higher than P- solubilization by PC4 for the same concentration of inoculum. It was recorded that P- solubilization by bacterial isolates increased significantly with increase in size of inoculum from 1% to 10% and decreased significantly thereafter. Also the maximum mean viable count (46.50×10^6 cfu/ml) was found for 10% size of inoculum and minimum mean viable count(21×10^6 cfu/ml) was found in case of 1% size of inoculum.Therefore, 10% size of inoculum was selected as the best size of inoculum for the P- solubilization and viable count by selected bacterial isolates. 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 in Table 11. 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, viable count, % growth inhibition against *Dematophora necatrix* (Plate 1) and % siderophore unit and PC4 is attaching significantly higher average value to final pH of supernatant. 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.

Molecular characterization (16S rDNA sequencing) of selected bacterial isolate 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 (Figure 1). The PCR product was eluted from gel, and sequenced using PCR primers. Dendogram based on phylogenetic analysis presented in (Figure 2) shows that *Bacillus* isolate (PkR(7a)*) is clustered with *Bacillus subtilis*. The sequence of respective isolate was submitted to NCBI under accession no. JN559852.

Discussion

Isolation, enumeration and identification of rhizobacteria and endorhizobacteria from medicinal plant *picrorhiza kurroa*

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 (Table 1 and 2).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) [23, 24]. 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 (Table1) 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) [25]. 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) [26-28]. 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) [29].

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) [30].

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) [31] and least correlation was found with P- solubilized and colony + halozone diameter (Alam *et al*., 2002 and Srivastav *et al*., 2004) [32, 33]. 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) [34, 35]. 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.

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) [38]. Tricalcium phosphate has been reported to be readily solubilized than rock phosphate (Srivastav *et al*., 2004) [33] and in the present study, TCP was selected as substrate for determining the phosphate solubilizing potential of selected bacterial isolates.

Four different media (PVK, NBRIP, LB and NB) were used in the study but due to the wide distribution of different *Bacillus* spp., PVK medium was selected for studying the culturable population of PSB, as previous study (Kundu *et al*., 2002 and Srivastav *et al*., 2004) [33] has shown this medium to be the most selective, least susceptible to overgrowth by non PSB and most reproducible, allowing the growth of a broad diversity of PSB. Using this medium, the differences in the number of total phosphate solubilizing bacterial colony forming units (cfu) was found significant between the different samples taken from different locations (Table 5).While comparing P-solubilization capacities of selected bacterial isolates in PVK broth, NBRIP broth, Luria Bertani broth and nutrient broth, it was observed that PVK broth proved to be better medium for P-solubilization. Higher solubilization of tricalcium phosphate observed in PVK medium (Table 5) is contrary to the results reported earlier where NBRIP supports more P-solubilization than PVK broth (Chatli *et al*., 2008) [29]. These results disprove the contention that NBRIP medium lacks yeast extract and contains lesser amount of ammonium sulphate ($(NH_4)_2 SO_4$) as compared to PVK medium,which are considered as non essential components in P-solubilizing medium (Nautiyal,1999). Therefore, NBRIP broth may not be considered a better medium for P-solubilization by isolates from *Picrorhiza kurroa*.

Also an inverse correlation (r=-0.70) was observed between pH and amount of P released in all the four media which was in agreement with the findings of (Kim *et al.,*1998) [40] 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) [41, 42].

The solubilization of TCP has been found to be highly dependent on temperature and pH. All the selected bacterial isolates showed maximum P- solubilization and viable count at temperature 35 ^{0}C and pH 7.0. Our finding is in support to the study (Gaur, 1990) [43] that bacteria *Bacillus polymyxa* and *Pseudomonas striata* showed maximum solubilization at pH 7 to 8 and *B. polymyxa* was found to have temperature range of 35 to 40 ^{0}C. This behavior of the bacterial isolates is similar to the usual response of mesophilic bacteria in which case, the metabolic activity get slow down below the optimum temperature and temperature above optimum, results in decrease in the viable count and impair metabolic activities through denaturation of certain enzymes involved in various metabolic processes.

Inoculum prepared from young culture of 24 h old was found most effective may be because the population in young culture is most nearly uniform in terms of biochemical and physiological properties in young culture. Therefore, exponential phase culture is usually used in biochemical studies. The increase in the P-solubilization and production of antibiotic activity with increase in incubation period is due in part to the increase in growth of organism. This is supported with our results where the P-solubilization increases with increase in the initial number of viable cells.

The P- solubilization was characterized by the initial rapid increase in P-solubilization in the culture medium at lower concentration of TCP, maximum at concentration 0.5 per cent and then followed by a decrease in P- solubilization with further increase in TCP concentration Viable count was also found to be maximum at TCP concentration 0.5 per cent and decreased significantly with further increase in TCP concentration. This may be because the rate of growth also increases with nutrient concentration, but in hyperbolic manner. At sufficient higher nutrient level, the transport systems are saturated, the growth rate decreases with increase in nutrient concentration when bacterial growth is limited by the low concentration of required nutrients (in this case available phosphorus). Addition of more TCP is not solubilized for want of organic acid molecules required for its solubilization. But there was no significant decrease in the final pH of the supernatant with increase in the TCP concentration showing that solubilization depends not only on the pH but also the structure and type of organic acid molecules and do not depend upon the organic acid concentration (Johnston, 1952) [44].

In the present study (Table 12), 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) [45]. 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) [46, 37, 47]. The optimum P-solubilization was observed at an incubation period of 72 h and our studies were in agreement with earlier reports (Nautiyal *et al*., 2000) [45].The decrease in soluble P at later incubation period of 96 h may be either due to decrease in solubilizing activity and increase in P absorption. The P concentration in culture medium followed a typical sigmoid curve unlike the increase and decrease of varying P concentration after different incubation time as reported by few authors (Illmer and Schinner, 1992, 1995) [26].

The phosphate solubilizing bacteria has been used since long as bioinoculants with promising results and if some PSB are found antagonistic to plant pathogen then combination of two attributes will be of great advantage in crop production. An attempt in this direction has been made during the present investigation.40.0 per cent inhibition of *Dematophora necatrix* by isolate PkR(7a)* with 20 per cent of culture filtrate corresponds to maximum P- solubilization (Table 12). The production of maximum antifungal antibiotic activity at the end of the exponential phase is in agreement with earlier studies on the production of peptide antibiotic that usually begin at late log phase of growth and continues with the stationary phase while working with *Bacillus megaterium* (Sultana *et al*., 2004) [48] and with *Bacillus* spp. (Sarkar and Paules, 1972 and Flio *et al*., 2004) [49, 50].

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) [51]. 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 (Table 12). The isolate PkR(7a)* produced siderophore, and had antifungal activity however, isolate PC4 neither produced siderophore nor had antifungal activity 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) [52, 53, 33] 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 [55] 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) [54].

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*.

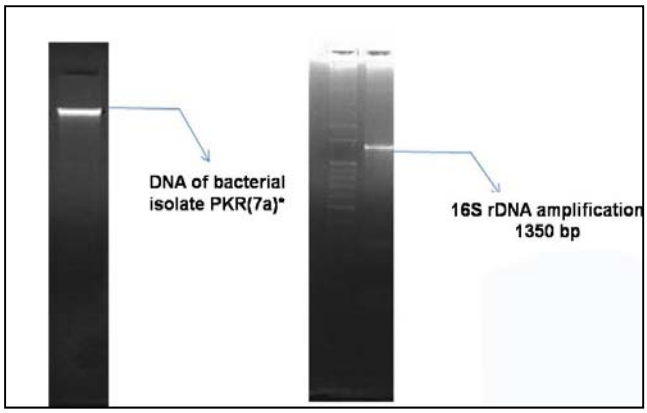

Fig 1: Gel photograph showing amplicon of 16S rDNA of *Bacillus subtilis* (PkR(7a)*) Lane 1:100 bp ladder ;Lane 2:1350 bp amplification

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

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

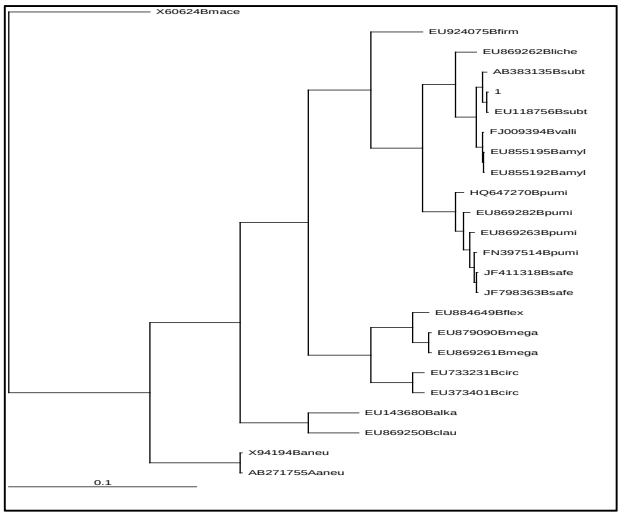

Fig 2: UPGMA phylogenetic tree based on 16S rDNA sequence data of *Bacillus subtilis* (PkR(7a)*) and related strains

Table 1: 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

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

Table 2: 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 (×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
*Same as in table 1

Table 3: Tricalcium phosphate solubilization (tcp) by selected bacterial isolates on solid media and liquid medium

Isolates	Phosphate solubilization	
	Phosphate solubilization index (PSI) **	Quatitative P-estimation (mg/l)●
Pk3A	2.45	105.00
Pk4a	2.27	135.00
PkH(4)	2.56	115.00
PkH(5)	2.61	130.00
Pk6(B)	2.88	120.00
Pk6(c)	2.50	100.00
Pk7(B)	2.58	120.00
Pk8(A)	2.35	135.00
Pk8(e)	2.43	95.00
Pk9(B)	2.50	95.00
Pk11(c)	3.00	125.00
Pk12(a)	2.50	75.00
Pk12(b)	2.80	50.00
Pk12(c)	2.43	85.00
Pk12(d)	2.38	100.00
Pk13(a)	3.16	95.00
Pk13(b)	2.80	80.00
Pk13(c)	2.56	120.00
Pk14(a)	3.00	90.00
Pk14(b)	4.05	205.00
Pk14(c)	3.42	40.00
PkR(2)*	2.36	80.00
PkR(21)*	2.40	100.00
PkR(22)*	2.32	55.00
PkR(32)*	2.54	110.00
PkR(33)*	2.33	55.00
PkR(34)*	2.35	70.00
PkR(5a)*	2.78	90.00
PkR(5e)*	2.62	90.00
PkR(6a)*	2.65	100.00
PkR(7a)*	2.73	320.00
PkR(7b)*	2.82	120.00
PkR(7c)*	3.30	90.00
PC2	3.00	80.00
PC3	2.75	90.00
PC4	2.60	180.00
PC7	3.07	75.00
PC8	2.66	80.00
PC9	3.05	50.00
PC13	3.42	60.00
$CD_{0.05}$	1.05	4.05

Table 5: Effect of different media on tricalcium phosphate solubilization by selected bacterial isolates after 72 h of incubation

Isolates	P-solubilization• (mg/l)				Mean	Viable count (×10⁶ cfu/ml)				Mean	Final pH of the supernatant				Mean
	Different media					Different media					Different media				
	PVK**	LB***	NBα	NBRIPαα		PVK**	LB***	NBα	NBRIPαα		PVK**	LB***	NBα	NBRIPαα	
PkR(7a)*	320.00	5.00	8.00	30.00	90.75	63.00	35.00	39.00	45.00	45.50	4.72	9.52	9.20	6.48	7.48
Pk14(b)	200.00	65.00	5.00	25.00	73.75	56.00	49.00	25.00	35.00	41.25	5.00	7.29	9.17	7.00	7.11
Pk12(d)	100.00	15.00	15.00	30.00	40.00	51.00	32.00	35.00	37.00	38.75	5.12	9.42	9.21	6.45	7.55
Pk7(B)	115.00	8.00	10.00	45.00	44.50	47.00	30.00	32.00	39.00	37.00	5.04	9.51	9.31	6.00	7.46
PC4	180.00	10.00	68.00	40.00	74.50	57.00	33.00	45.00	47.50	45.62	5.05	9.48	7.30	6.19	7.00
Mean	183.00	20.60	21.20	34.00		54.80	35.80	35.20	40.70		4.98	9.04	8.83	6.42	
									r = - 0.70						
$CD_{0.05}$															
	Isolates (I)				2.15					1.73					0.70
	Media(M)				1.92					1.54					0.63
	I × M				4.30					3.46					1.39

* Endorhizobacteria
**Pikovskaya's Broth,
***Luria Bertani Broth,
α Nutrient Broth,
ααNational Botanical Research Institute Phosphate Growth Media
● T- C ; Where, T = Inoculated PVK with TCP, C (Uninoculated PVK with TCP)

Table 6: Effect of incubation period on tricalcium phosphate solubilization by selected bacterial isolates in Pikovskaya's broth medium

Isolates	P-solubilization• (mg/l)				Mean	Viable count (×10⁶ cfu/ml)				Mean	Final pH of the supernatant				Mean
	Incubation period					Incubation period					Incubation period				
	24	48	72	96		24	48	72	96		24	48	72	96	
PkR(7a)*	180.00	220.00	320.00	300.00	255.00	29.00	37.00	46.00	43.00	38.75	4.92	4.87	4.72	4.88	4.84
PC4	50.00	90.00	185.00	170.00	123.75	27.00	36.00	40.00	37.00	35.00	5.34	5.19	5.01	5.13	5.16
Mean	115.00	155.00	252.50	235.00		28.00	36.50	43.00	40.00		5.13	5.03	4.86	5.00	
$CD_{0.05}$															
	Isolates (I)				2.52					2.19					0.23
	Incubation period (P)				3.56					3.10					0.33
	I × P				5.04					4.36					0.46

* Endorhizobacteria
●T- C : Where, T = Inoculated PVK with TCP, C (Uninoculated PVK with TCP)

Table 7: Effect of age of inoculum on tricalcium phosphate solubilization by selected bacterial isolates in Pikovskaya's medium after 72 h of incubation

Isolates	P-solubilization• (mg/l)					Mean	Viable count (×10⁶ cfu/ml)					Mean	Final pH of the supernatant					Mean
	Age of inoculum (h)						Age of inoculum (h)						Age of inoculum (h)					
	24	48	72	96	120		24	48	72	96	120		24	48	72	96	120	
PkR(7a)*	320.00	310.00	230.00	220.00	215.00	259.00	47.00	41.00	35.00	33.00	29.00	37.00	4.67	4.75	4.80	4.84	4.95	4.80
PC4	170.00	135.00	105.00	100.00	70.00	116.00	43.00	37.00	31.00	30.00	25.00	33.20	5.05	5.08	5.14	5.16	5.20	5.12
Mean	245.00	222.50	167.50	160.00	142.50		45.00	39.00	33.00	31.50	27.00		4.86	4.91	4.97	5.00	5.07	
Temperature 35 ºC pH 7.0																		
$CD_{0.05}$																		
	Isolates (I)					2.83						2.12						0.46
	Age of inoculum (A)					4.49						3.36						0.73
	I × A					6.34						4.75						1.02

* Endorhizobacteria
●T- C : Where, T = Inoculated PVK with TCP, C (Uninoculated PVK with TCP)

Table 8: Effect of size of inoculum on tricalcium phosphate solubilization by selected bacterial isolates in Pikovskaya's medium after 72 h of incubation

Isolates	P-solubilization• (mg/l)				Mean	Viable count (×10⁶ cfu/ml)				Mean	Final pH of the supernatant				Mean
	Size of inoculum (%)					Size of inoculum (%)					Size of inoculum (%)				
	1	5	10	15		1	5	10	15		1	5	10	15	
PkR(7a)*	260.00	290.00	320.00	300.00	292.50	20.00	31.00	48.00	36.00	33.75	4.90	4.80	4.73	4.78	4.80
PC4	135.00	155.00	185.00	155.00	157.50	23.00	28.00	45.00	33.00	32.25	5.40	5.35	5.01	5.14	5.22
Mean	197.50	222.50	252.50	227.50		21.50	29.50	46.50	34.50		5.15	5.07	4.87	4.96	
Temperature 35 ºC pH 7.0 Age of inoculum 24 h															
$CD_{0.05}$															
	Isolates (I)				3.70					2.20					0.22
	Size of inoculum (S)				5.24					3.12					0.32
	I × S				7.42					4.42					0.44

* Endorhizobacteria
●T- C : Where, T = Inoculated PVK with TCP, C (Uninoculated PVK with TCP)

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