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ORIGINAL PAPER

Solubilization of organic and inorganic phosphates by three highly efficient soil bacterial isolates

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Abstract Screening soil samples collected from a diverse range of slightly alkaline soil types, we have isolated 22 competent phosphate solubilizing bacteria (PSB). Three isolates identified as *Pantoea agglomerans* strain P5, *Microbacterium laevaniformans* strain P7 and *Pseudomonas putida* strain P13 hydrolyzed inorganic and organic phosphate compounds effectively. Bacterial growth rates and phosphate solubilization activities were measured quantitatively under various environmental conditions. In general, a close association was evident between phosphate solubilizing ability and growth rate which is an indicator of active metabolism. All three PSB were able to withstand temperature as high as 42°C, high concentration of NaCl upto 5% and a wide range of initial pH from 5 to 11 while hydrolyzing phosphate compounds actively. Such criteria make these isolates superior candidates for biofertilizers that are capable of utilizing both organic and mineral phosphate substrates to release absorbable phosphate ion for plants.

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Introduction

As the world non-renewable resources of extractable phosphorus (P) is becoming depleted and natural ecosystems are impaired by the build up of phosphorus and associated heavy metals progressively, exploring the alternative approaches for retrieving P from low-profile P rocks has become a necessity. With regard to the fact that the majority of purified P as phosphoric acid is used in agriculture in the form of various P chemical fertilizer types, one of the most feasible strategies toward environmentally sustainable agriculture is to seek out microbial inoculants to salvage P compound in place. This has lead researchers to establish screening methods for the isolation of phosphate solubilizing bacteria (PSB) from soil and subsequent evaluations for specific criteria particularly with respect to applications in harsh environmental conditions (Morrissey et al. 2004).

In the field, P is the second most essential element for plant growth and development that is absorbed only in soluble forms of phosphate ion (P_i), HPO_4^{2-} or $H_2PO_4^-$. In fact, total soil phosphorus content is well beyond plant needs (400–1,200 mg/kg); however, it is mostly immobilized in the forms of organic and inorganic compounds such that the concentration of soluble P_i is well below 1 ppm (for a review see Rodriguez and Fraga 1999). Even, added chemical P_i fertilizer is rapidly fixed in soil to its insoluble forms. Therefore, the availability of P_i is highly dependent on chemical compositions and biological processes occurring in the soil, particularly within the

rhizospher microenvironment (Illemer and Schinner 1995; Illmer et al. 1995; Garcia et al. 1997).

It has already been shown that the use of PSB as biofertilizers assists the hydrolysis of a wide range P compounds leading to higher crops yields and reduce chemical hazards to the environment (for reviews see Rodriguez and Fraga 1999; Igual et al. 2001). In this regard, the isolation of PSB from native soils is favored as such bacteria are well adapted to the natural climate of their habitat (Johri et al. 1999; Son et al. 2006).

The objective of this research was to isolate PSB strains capable of hydrolyzing both organic and inorganic P_i compounds more effectively. Here, we have reported the isolation of several PSB from alkaline soil samples. As a new approach, we have set up a method for screening of PSB with high phosphatase activity. The effects of several environmental conditions on propagations and P_i solubilizing activities of the isolates were assayed.

Materials and methods

Isolation of PSB strains and comparisons

Around 200 samples for cultivated and non-cultivated soils with pH 7–8 were removed from the rhizosphere of crop or wild plants and transferred to laboratory in sterile capped containers. To screen for PSB, one gram of each soil sample was suspended in 9 ml of sterilized ddH_2O and mixed vigorously. Serial dilutions were prepared and plated on Sperber medium (Rao et al. 1982) composed of 10 g glucose, 0.5 g yeast extract, 0.1 g $CaCl_2$, 0.25 g $MgSO_4 \cdot 7H_2O$ supplemented with 2.5 g $Ca_3(PO_4)_2$ and 15 g agar per liter. After 48 h incubation at room temperature, plates were examined for clear zones around bacterial colonies. The PSB colonies were picked and subcultured as above until a pure culture was obtain. Soil samples were also screened for phosphatase activity by plating on the Sperber plates containing 50 mg/l 5-bromo-4-choloro-3-indolyl phosphate (BCIP; Sigma, St. Louis, Mo). Incubating at room temperature overnight, blue-stained colonies were picked and purified as above.

To compare P_i solubilization capabilities, 25 µl of bacterial suspension diluted to 10^8 CFU/ml was spotted on the center of solid Sperber medium plate containing insoluble P_i. The diameter of clear zone around each colony was measured after 1, 2 or 4 days, in triplicate. Phosphatase activities in isolates were compared based on scoring the intensities of stained colonies in days 1, 2 and 7 in the presence of soluble phosphate (KH_2PO_4) or insoluble phosphate, $Ca_3(PO_4)_2$.

Identification of the isolates

Three selected PSB isolates were identified by biochemical tests including gram staining, utilization of citrate and different sugars, gelatin hydrolysis, oxidative/fermentative test (O/F), H_2S and indole production, anaerobic growth, motility, methyl red test, Voges–Proskauer (V–P) reaction as well as the activities of catalase, oxidase, lecetinase, amylase, urease (Palleroni 1984; Sneath 1986). 16S rDNA amplification and sequencing were performed according to Morabbi Heravi et al. (2008). rDNA gene amplifications of bacterial strain P5 and P7 were carried out using oligonucleotide primers fD1 (5′-AGA GTT TGA TCC TGG CTC AG-3′) and rP1 (5′-ACG GTT ACC TTG TTA CGA CTT-3′) Weisburg et al. (1991). For strain P13, oligonucleotide primers fD1 and rD1 (5′-TAA GGA GGT GAT CCA GC-3′) were used Weisburg et al. (1991). The amplified PCR fragments were cloned into pTZ57R/T vector (Fermentas) prior to sequencing. Sequences similarities were analyzed using GenBank database and BLASTN software at http://www.ncbi.nih.gov/blast (Altschul et al. 1997) as well as Ribosomal Data Project and tools at http://rdp.ome.msu.edu (Cole et al. 2008).

Bacterial growth and phosphate solubilization assessments

Time-coursed quantitative measurements were carried out in 120 ml Erlenmeyer flasks containing 15 ml of Sperber broth medium inoculated with $\sim 10^4$ CFU/ml of each bacterial strain and incubated at 30°C and 120 rpm. The same non-inoculated medium served as control in each case. Deviations from the above growth conditions were as below. The effect of temperature was examined by incubation at 25, 35 and 42°C. For high-salt treatments, the extra amounts of salt (0.5, 1, 2.5 and 5%; w/v) were added to Sperber medium. Initial pH values of medium ranging from 5 to 11 were adjusted by the addition of either KOH or HCl accordingly (see Results). Sampling within 48 h, the growth rate was estimated by counting colonies on solid Sperber medium while the measurement of released P_i in culture supernatant was estimated by Fisk and Sabbarow method (Fisk and Sabbarow 1925). Data analyses and graph drawing were all done by the use of Microsoft Excel program.

Results

Having screened alkaline soil samples, 22 promising PSB isolates were selected that produce large clear zones or blue color in minimal medium containing insoluble P_i or synthetic substrate, respectively, within the first 2 days of

plating. In a comparative experiment set up, the largest clear zone was observed for P5 (21 cm) followed by P7 (18 cm) within the first day (Table 1). Also, the strongest blue color was developed by P13 and then P10 and P14 within the first day while the others stained weakly or not at all (Table 1). Two PSB with the largest clear zone, P5 and P7, and one with the highest phosphatase activity, P13, were chosen for further characterization. Several biochemical and biological indicators as well as 16S rDNA sequence were inspected to figure out the taxonomy of these isolates. The compiled data (not shown) revealed that P5, P7 and P13 isolates must be strains of *Pantoea agglomerans*, *Microbacterium laevaniformans* and *Pseudomonas putida* species, respectively. 16S rDNA sequences of these isolates (GenBank accession Nos. EU545412, EU545413 and EU545414) were all in agreement with the biochemical tests.

In a series of preliminary experiments, the effects of various growth conditions on the survival of the selected PSB were assessed. *P. putida* was capable of growing on solid medium with added salt up to 2.5% and at temperature below 40°C. In contrast, *P. agglomerans* and *M. laevaniformans* were capable of forming colonies at temperature up to 45°C, pH up to 9 and salt as high as 7% when grown on solid medium (data not shown).

This study was detailed furthermore by using liquid minimal medium for which two indices were monitored: (1) growth index (GI) which is the logarithm of CFU/ml of culture; and (2) P_i solubilizing index (PSI) that repersents the amounts of hydrolyzed P_i from tricalcium phosphate substrate. Almost in all cases, a close association between GI and PSI was noticeable (Figs. 1, 2, 3). The maximum levels of both GI and PSI for *P. agglomerans* was reached after 12 h of incubation at 25°C while exponential growth phase of P13 delayed for 6 h and arrived at stationary phase after 24 h. Despite that, a higher PSI was obtained for P13 after 48 h (150 mg/l) in compare to P5 and P7 (about 120 mg/l) after 12 h (Fig. 1). Generally, bacterial growth and consequently PSI of all bacteria were reduced at high temprature (Fig. 1). This was more pronounced for

Table 1 An inventory of the isolated PSB from alkaline soil samples for which a summary of observed Gram reaction, bacterial shape, produced clear zone and presence or absences of phosphatase activity are given

Bacterial strain	Gram stain	Shape[a]	Diameter of clear zone (mm)[b]			Phosphatase activity					
			24 h	48 h	96 h	With soluble P_i			With insoluble P_i		
						24 h	48 h	7 days	24 h	48 h	7 days
P1	–	B	11.0 ± 0.6	14.3 ± 0.3	15.0 ± 0.0	–	–	–	–	–	–
P2	–	B	14.3 ± 0.9	15.3 ± 1.5	18.0 ± 1.2	–	–	–	–	–	–
P3	–	C	0	0	16.3 ± 1.4	–	–	+	–	–	–
P4	–	B	12.0 ± 0.6	13.3 ± 0.3	14.3 ± 0.3	–	–	–	–	–	–
P5	–	B	21.0 ± 3.5	22.0 ± 4.1	21.7 ± 4.1	–	+	+	+	+	+
P6	+	B	13.7 ± 2.1	16.0 ± 2.1	14.7 ± 2.1	–	–	–	–	–	–
P7	–	B	18.0 ± 3.9	19.3 ± 3.5	18.0 ± 3.5	–	+	+	+	+	+
P8	+	B	0	12.7 ± 0.3	14.3 ± 0.7	–	–	–	–	–	–
P9	+	B	12.0 ± 0.6	15.3 ± 0.3	17.7 ± 1.2	–	–	–	–	–	–
P10	+	B	12.3 ± 0.3	14.3 ± 0.9	16.7 ± 1.2	+	+	+	+	+	+
P11	–	B	12.3 ± 0.3	15.7 ± 0.9	18.3 ± 0.3	–	–	–	–	–	–
P12	+	B	13.0 ± 0.8	14.0 ± 1.0	16.0 ± 0.6	–	–	–	–	–	–
P13	–	CB	11.0 ± 0.6	13.3 ± 0.3	14.0 ± 0.6	–	–	–	+	+	+
P14	–	B	9.3 ± 0.3	12.7 ± 0.3	16.0 ± 0.0	+	+	+	–	+	+
P16	–	B	0	0	0	–	–	+	–	–	–
P17	–	B	11.7 ± 0.7	15.0 ± 0.6	16.3 ± 1.5	–	–	–	–	–	–
P18	+	C	13.3 ± 1.9	14.3 ± 0.7	15.7 ± 0.9	–	–	–	–	+	+
P19	+	B	8.7 ± 4.5	9.0 ± 4.6	13.7 ± 1.5	–	–	–	–	–	–
P20	+	B	0	0	0	–	–	–	–	+	+
P21	+	B	11.7 ± 0.9	14.7 ± 0.9	17.0 ± 2.1	–	–	+	–	+	+
P23	–	B	13.3 ± 0.7	20.7 ± 0.7	26.0 ± 1.2	–	–	–	–	–	–
P24	–	B	15.0 ± 0.6	19.0 ± 0.6	23.0 ± 0.6	–	–	–	–	–	–

[a] Bacterial shapes: B, Bacillus; C, Coccus; CB, Coccobacillus

[b] The figures are means of three replicates ± standard errors

P13 strain such that it never entered exponential growth phase when incubated at 42°C, although they were alive. Interestingly, a higher PSI value was obtained for P13 (73 mg/l) after 12 h of incubation at 35°C. This was gradually decreased when incubation was extended beyond 24 h.

As shown in Fig. 2, the additions of NaCl upto 2.5% did not affect GI of the PSB isolates for the first 12 h. However, PSI values for P5 was inversely proportional to the added salt, particularly when the added salt was higher than 2.5%. A different pattern was observed for P13 strain; P_i solubilization was delayed in low salt medium and the highest PSI value at 12 h sampling was obtained in medium with 1% added salt. As expected, both GI and PSI values were reduced when the amount of added NaCl was increased to 5%.

Inoculation into medium with various initial pH did not affect the GI of all the isolates, except for P7 in acidic broth when incubation was extend for more than a day (Fig. 3). However, PSB isolates performed differently in terms of PSI. The PSI values for P5 and P7 were the highest at pH 9 and 10, while that was the highest in acidic pH for P13 strain. It is noteworthy that within the culturing period pH of the medium is reduced to around three by the bacteria (data not shown) resembling what happens in microenvironment across the bacterial film in rhizosphere.

Discussion

Beneficial bacteria, collectively called plant growth promoting rhizobacteria (PGPR), are the main constituents of

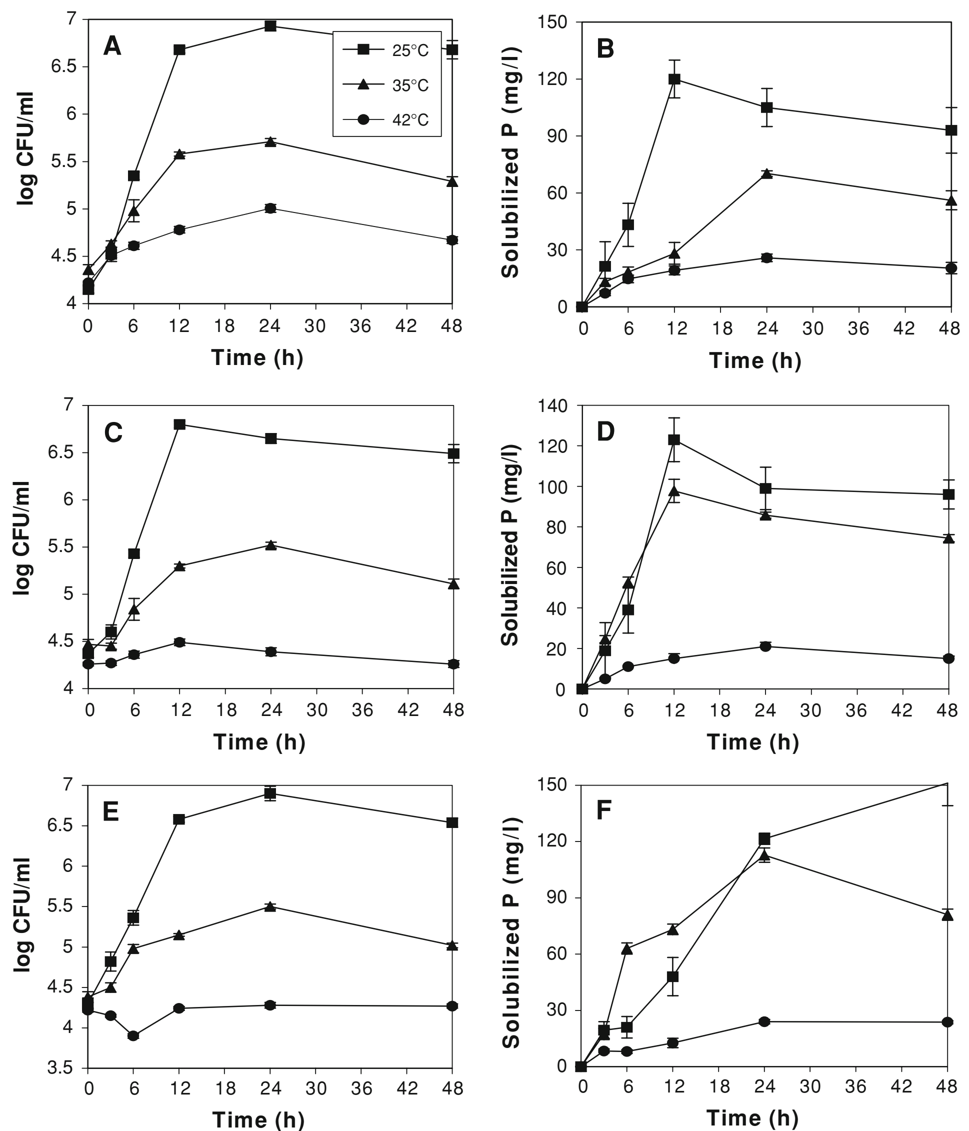

Fig. 1 The effect of different incubation temprature on the bacterial growth (*panels A*, *C* and *E*) and the P_i solubilzation (*panels B*, *D* and *F*) rates. Bacterial strains P5 (*panels A* and *B*), P7 (*panels C* and *D*) and P13 (*panels E* and *F*) were grown in liquid medium containing tricalcium phosphate. All data points are the means of three replicates. Standard errors are shown by *vertical* bars

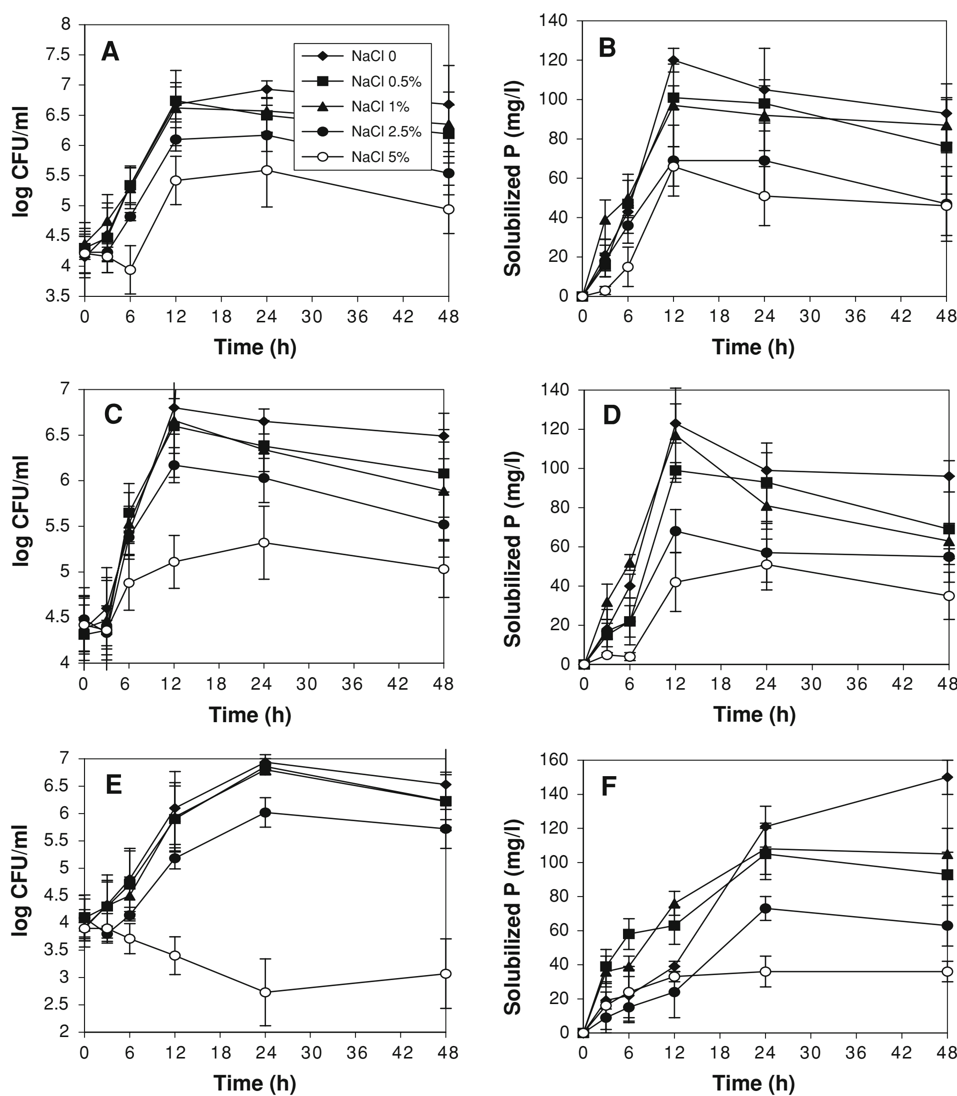

Fig. 2 The effect of various NaCl amounts added to minimal liquid medium on bacterial growth and P_i Solubilzation. Panels are arranged as Fig. 1. All data points are the means of three replicates. Standard errors are shown by *vertical* bars

biofertilizers. These bacteria promote plant growth and health by various means such as mineralization of nutritional elements, synthesizing phytohormones, suppressing pathogens or combinations of them (for a review see Somers and Vanderleyden 2004). Nevertheless, the performance of PGPR as biofertilizers is severely influenced by both biotic and abiotic environmental conditions of the targeted regions. This has lead researchers to seek out isolation of PGPR from native soil and to explore methods for screening PGPR strains and subsequent evaluations for specific criteria particularly with respect to applications in various environmental conditions.

As one of the very important group of PGPR for sustainable agriculture, PSB tolerances to extreme climates are of special interests for bacteria to be used as biofertilizer in arid and semi-arid regions. Thus, the isolates were intensively examined for tolerance toward high temperature, high concentration of NaCl and a wide range of pH (Figs. 1, 2, 3). All PSB strains survived at high temperature very well. Decreased GI and PSI at 42°C for the isolated PSB are acceptable as in natural situation these bacteria survive hot noon of summer time and continue growing on the rest of the days. Givskov et al. (1994) also showed that *P. putida* survives several hours of incubation at 45°C. All PSB could tolerate added NaCl to as much as 2.5% which indicate that these isolates would be functionally active in most cultivated lands where salinity of soil is well below 2.5%. It is noteworthy that high salt concentrations increase buffering capacity of medium that may cause reductions in PSI to some degrees. Enhanced PSI of P13 strain at 35°C and in salt concentrations between 0.5 and 1% may suggest induced acid production in moderately stressed cells (Fig. 2). As a key feature of cultivation soils, we measured GI and PSI of bacteria grown in medium with various initial pH ranging from 5 to 11. The fact that the highest GI and PSI values for *P. agglomerans* and *M. laevaniformans* were obtained at high pH suggests that these bacteria favor alkaline condition. There was no differential lag in the initial growth rate of both

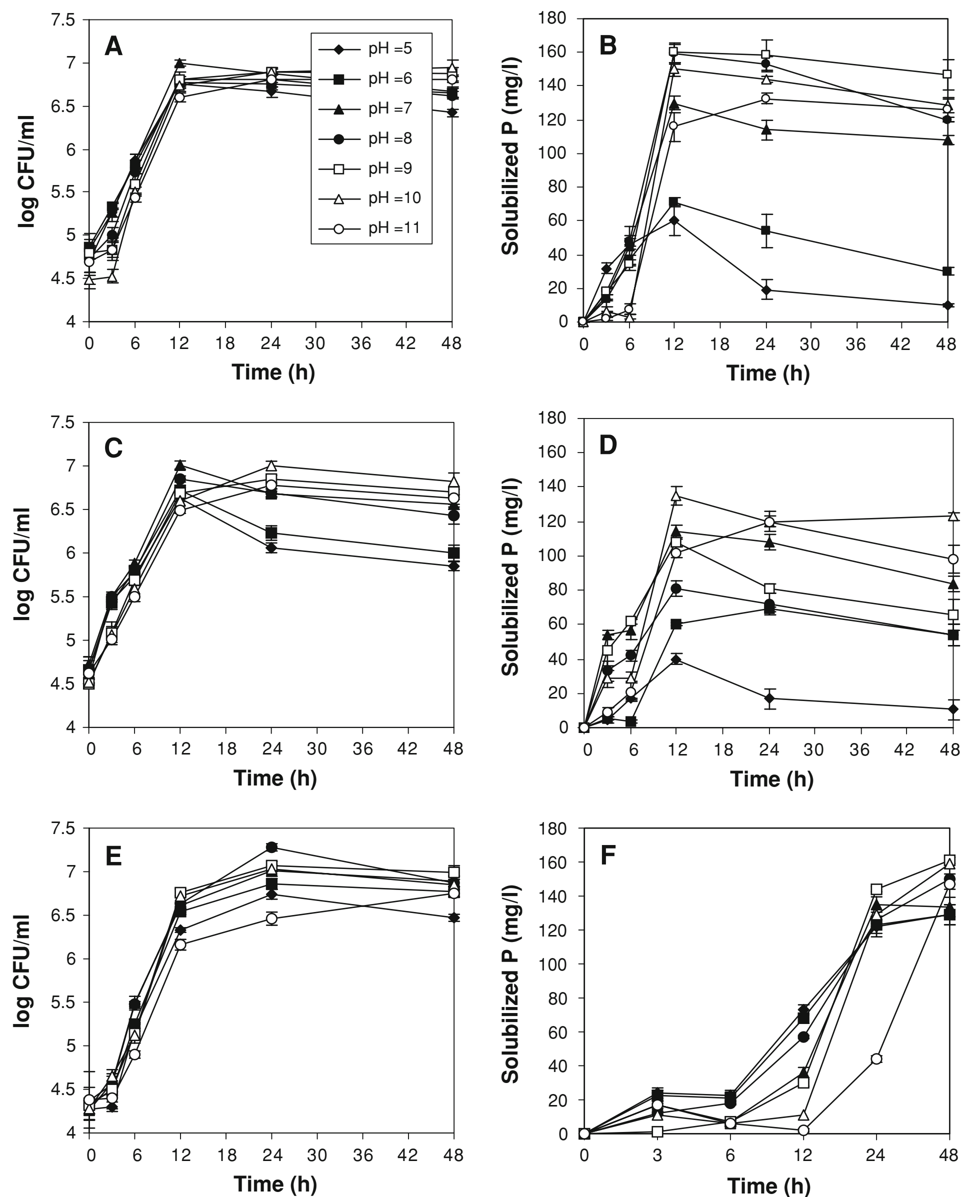

Fig. 3 The effect of different initial pH of medium on bacterial growth and P_i solubilzation. Panels are arranged as Fig. 1. All data points are the means of three replicates. Standard errors are shown by *vertical* bars

bacterial strains while at acidic pH GI was significantly lower for the latter, although the broth was not buffered. Interestingly, a 6–12 h lag in the increase of PSI was observed for P13 strain which is believed to produce acid phosphatase enzymes with optimal activity in acidic pH.

In a similar study, Nautiyal et al. (2000) investigated the effects of pH, temperature and salt on four PSB isolated from pea rhizosphere in India. Even though no data was presented for GI, they showed that all the four examined strains efficiently solubilized P_i at 5% NaCl. One of the isolates was capable of hydrolyzing P_i even in the presence of 10% NaCl at the rate of 18% compare to the control bacterial culture. In another study, Johri et al. (1999) surveyed 4,800 bacterial strains isolated from alkaline soil. They found 18 out of 857 PSB isolates could efficiently solubilize P_i in high levels of pH, temperature and salt. Also, Son et al. (2006) isolated *P. agglomerans* strain R-42 with maximal production of 1.3 g/l soluble phosphate in an optimized medium. This isolate also showed tolerance toward different environmental factors such as pH (3–11), temperature (5–45°C) and 5% NaCl (Garcia et al. 1997). This species is also known as a biocontrol agent against decaying fungi during storage of fruits (Garcia et al. 1997) and fire blight, a severe tree disease (Sandra et al. 2001; Hector et al. 2008).

It is strongly suggested that PSB take advantage of their organic acid exudates such as citrate, oxalate, glucanate, lactate and succinate (Igual et al. 2001; Rodriguez and

Fraga 1999). However, as the activity is much higher than what is expected from the effect of organic acids, some authors raised reasonable doubt about the real cause. Illemer and Schinner (1995) speculated that the release of protons accompanying respiration or NH_4 assimilation may play the major role in P_i solubilization mechanism, at least for the studied microorganism. Consistently, we have observed that the P_i solubilization is a function of active bacterial growth (Figs. 1, 2, 3). A noticeable reduction in PSI during stationary phase of growth found in all cases also supports the dependence of PSI on bacterial metabolism. Additionally, it was shown that the phosphatase activity of P13 bacterial strain could synergistically enhance the release of P_i in the acidified medium (Fig. 3).

In conclusion, we have isolated several PSB strains, three of which were more promising for being used as P_i biofertilizers. These bacteria are well adapted to various environmental conditions. As shown by Malboobi et al. (2009) the competitiveness of these bacteria and their effect on higher biomass production and yield are shown in both greenhouse and field trials.

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