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In vitro solubilization of inorganic phosphates by phosphate solubilizing microorganisms

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Bacterial strains isolated from green house soils were incubated *in vitro* for seven days to assess their phosphate solubilizing capacity, solubilization index, organic acid production and acid and alkaline phosphatase activity. All the tested isolates solubilized inorganic phosphates in varying degrees in NBRIP liquid culture medium. Acidification by means of producing organic acids may be the key mechanism attributed to increased phosphate solubilization as revealed by the strong negative correlation (r = - 0.923 ± 0.1, P ≤ 0.05) observed between the amount of phosphorus released and pH in the culture medium. Moreover, acid and alkaline phosphatase released by the isolates may also have certain contribution to phosphate solubilization. Gluconic acid was the most common acid produced by isolated organisms followed by citric and oxalic acid. Out of the isolates, strains PSB–1(*Pantoea agglomerans*), PSB–5 (*Burkholderia anthina*) and PSB–8 (*Enterobacter ludwigii*) solubilized significantly higher amount of inorganic phosphate within a day, exhibiting their higher potential to be used as soil inoculants to enhance soil fertility and plant growth.

Key words: Organic acids, phosphatase activity, solubilization index, phosphate solubilization.

INTRODUCTION

It has been evident that the world agriculture heavily depends on synthetic fertilizers as the source of plant nutrients to meet the increasing demand for food. However, continued and unabated use of chemical fertilizers often results in unexpected negative impacts on the environment. Imbalanced soil microbial activity and poor soil fertility could also result from unlimited use of chemical fertilizers. Thus, increasing attention is paid on the use of organics including biofertilizer to sustain the soil fertility and plant productivity.

The majority of agricultural soils contains large reserves of phosphorous, a considerable part of which has accumulated as a result of regular applications by field practitioners to alleviated phosphorus fertilizer problems (Richardson, 2004). A large portion of inorganic phosphate applied to soil as chemical fertilizer is rapidly immobilized and becomes unavailable to plants. Thus, the release of insoluble and fixed forms of phosphorus is an important aspect of increasing soil phosphorus availability.

A diverse group of soil microflora is reported to be involved in solubilizing insoluble phosphate complexes supplying plants with available phosphorus especially in soils with limited phosphorus (Tripura et al., 2005). Microorganisms that convert insoluble phosphates into soluble forms are termed phosphate solubilizing microorganisms. Solubilization is achieved through acidification, chelation, ion exchange reactions and production of low molecular weight organic acids such as gluconic, oxalic and citric acids (Chaiharn and Lumyong, 2009). In addition to providing available phosphorus to plants, phosphate solu-

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bilizing microorganisms can enhance plant growth through several different mecha-nisms, such as symbiotic nitrogen fixation, ammonia production, production of plant hormones and control of phytopathogenic microorganisms (Rangarajan et al., 2003). Therefore, the use of organisms with higher phosphate solubilizing abilities has proved to be an economically sound alternative to the more expensive superphos-phates and thus posses a greater agronomic utility (Khan et al., 2007).

In the present study, we isolated efficient phosphate solubilizing microorganisms from various green house soils to assess their phosphorus releasing capacity from inorganic phosphate sources.

MATERIALS AND METHODS

Isolation of strains

Soils used in this study to isolate phosphate solubilizing microorganisms were locally collected from green houses in Gongju-Gun area of Chungchugnam-do province, South Korea. Soil samples of 10 g were transferred into 250 ml Erlenmeyer flasks containing sterilized 90 ml of 0.85% NaCl solution. The mixture was then shaken for 30 min at approximately 150 rpm. Immediately after shaking, a series of tenfold dilutions of the suspension was made by pipetting 1 ml aliquots into sterilized 9 ml of 0.85% NaCl solution. Aliquots of 0.1 ml of the sample from each of these dilutions were spread on to a Petri dish with National Botanical Research Institute Phosphorus (NBRIP) medium containing 10 g of glucose, 5 g of $Ca_3(PO_4)_2$, 5 g of $MgCl_2.6H_2O$, 0.25 g of $MgSO_4.7H_2O$, 0.2 g of KCl and 0.1 g of $(NH_4)_2SO_4$ in 1 L distilled water (Nautiyal, 1999). The pH of the media was adjusted to 7 using HCl. The plates were incubated for 7 days in an incubator at 30°C. The colonies with clear halos were considered to be phosphate solubilizing colonies. Predominant colonies were further purified by re-streaking on the fresh NBRIP agar plates at 30°C. Five bacterial strains from those exhibiting large clear zones on the agar plates were selected as phosphate solubilizing organisms for further studies.

Strain identification

The partial sequencing of 16S rRNA for the bacterial strains was done with the help of DNA sequencing service, SOLGENT, Daejeon, South Korea using universal primers, 27F (5'-AGAGTTTGATCCTGGCTCAG -3') and 1492R (5'-GGTTACCTTGTTACGACTT -3') and PCR was performed with initial denaturation at 95°C for 2 min followed by 30 cycles with denaturation for 30 s at 94°C, annealing for 30 s at 58°C and extension for 45 s at 72°C. Final extension was held for 5 min at 72°C. The online program BLAST was used in identifying the related sequences with known taxonomic information available at the databank of NCBI (http://www.ncbi.nlm.nih.gov/BLAST). A phylogenetic tree was constructed using CLUSTAL X program (Thompson et al., 1997), which involved sequence alignment by neighbor joining method (Saitou and Nei, 1987) and maximum parsimony using the MEGA4 program (Kumar et al., 2001). Grouping of sequences was based on confidence values obtained by bootstrap analysis of 1,000 replicates. Gaps were edited in the BioEdit program and evolutionary distances were calculated using Kimura two parameter model. Reference sequences were retrieved from GenBank under the accession numbers indicated in the trees (Figure 1).

Solubilization Index

A pin point inoculation of each bacterial strain preserved in sterilized 30% glycerol was placed on NBRIP agar plates (n = 3) under aseptic conditions and incubated at 30°C for 7 days. Solubilization index was calculated daily using following formula (Edi-Premono et al., 1996).

$$\text{Solubilization Index} = \frac{\text{Colony diameter + halo zone diameter}}{\text{Colony diameter}}$$

Assay of inorganic phosphate solubilizing ability

Bacterial strains were grown in sterilized liquid NBRIP medium (20 ml) at 30°C for 2 days with continuous shaking at 150 rpm. Aliquots of culture (1 ml) was then transferred into a 500 ml flask containing sterilized liquid NBRIP medium (200 ml) and incubated for 7 days with continuous shaking at 30°C. Sterilized uninoculated medium served as a control. Aliquot (10 ml) of each culture and control was taken daily and centrifuged at 8000 rpm for 15 min. The clear supernatant was used in determining the amount of phosphorous released into the medium. The pH of the culture medium was also recorded with a pH meter equipped with glass electrode. Phosphorus availability was determined using phospho-molybdate blue color method (Murphy and Riley, 1962).

Assay of acid and alkaline phosphatases activity

Acid and alkaline phsosphatase activities were determined using a modified assay of Juma and Tabatabai (1988). Aliquot of centrifuged culture supernatant (1 ml) was incubated at 37°C with 1 ml of 25 mM p-nitrophenyl phosphate (pNPP) and 4 ml of modified universal buffer (pH adjusted to 6.5 and 11, respectively for acid and alkaline phosphatase assay). After 1 h, the reaction was terminated by adding 1 ml of 0.5 M $CaCl_2$ and 4 ml of 0.5 M NaOH. The assay mixtures were filtered and spectrophotometer readings were taken at 410 nm to quantify the intensity of yellow color (Tabatabai, 1982). Amount of released p-nitrophenol (pNP) was quantified using the pNP standard and expressed in terms of units (U). One unit (1 U) of phosphatases activity is the amount of enzyme required to release 1 µg pNP in 1 ml of culture filtrate under assay conditions (1U = 1 µg pNP/ml) (Prasanna et al., 2011).

Assay of organic acid production

To determine the organic acid composition of the different cultures, aliquots from the supernatants were analyzed using high-performance liquid chromatography (HPLC). A column Inertsil ODS 3V was used with a UV detector set to 210 nm at 40°C. Mobile phase consisted of 0.008 M H_2SO_4 run at a flow rate of 0.2 ml/min. HPLC profiles of the culture filtrates were analyzed by comparison with the elution profiles of pure organic acids (gluconic acid, oxalic acid and citric acid) injected separately. Peaks were identified by retention times against a set of standards from the three known organic acids.

Statistical analysis

The data were subjected to analysis of variance (ANOVA) using SAS package (SAS, 1999). The Duncan's Multiple Range Test (DMRT) was applied to test the significance of treatment means at $P \leq 0.05$. Each of the above tests was based on two or three repeat experiments, each containing three replications.

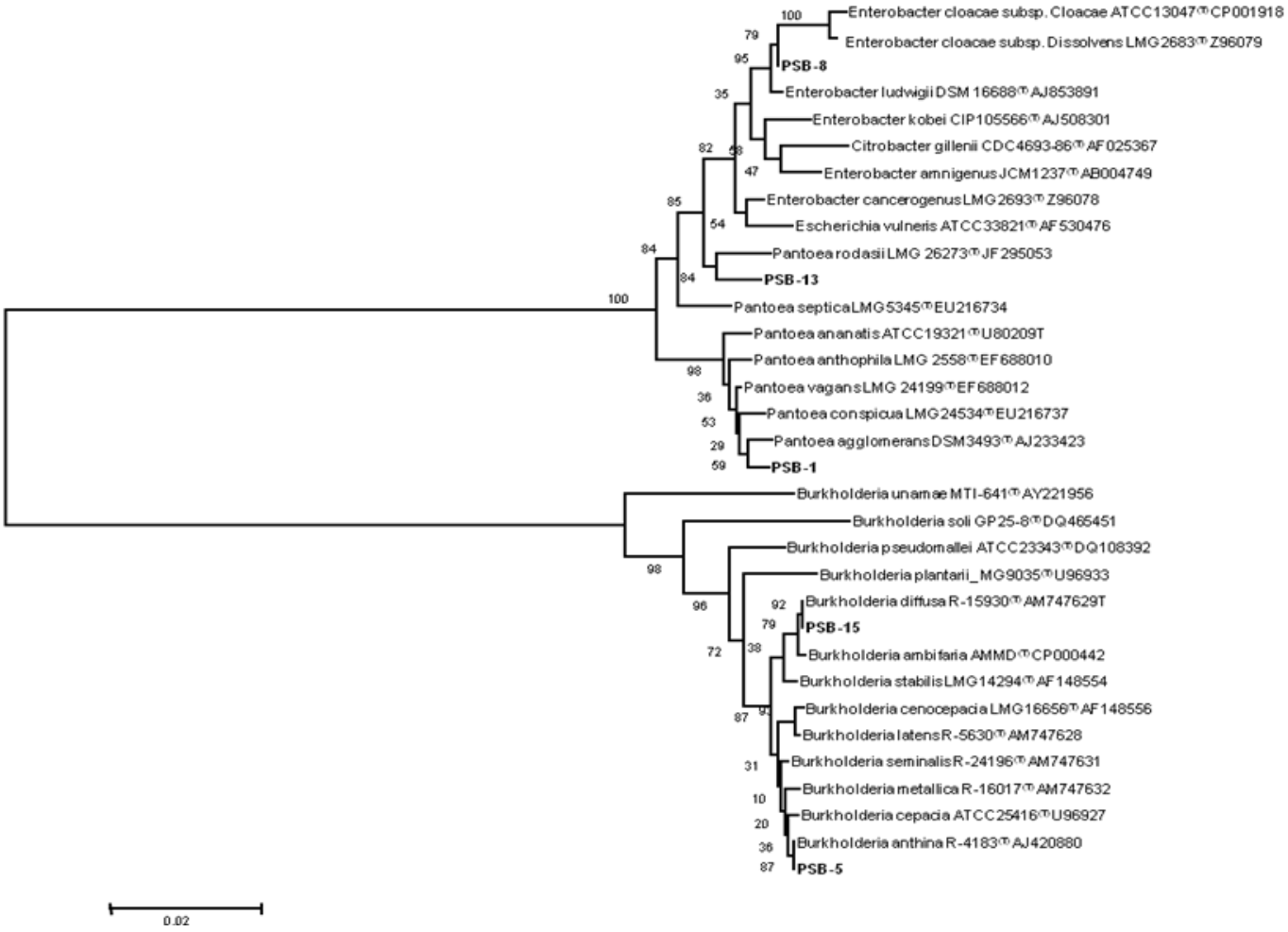

Figure 1. Phylogenetic tree (based on 16S rDNA gene sequences) showing the position of isolated phosphate solubilizing bacterial strains with respect to related species. The scale bar indicates 0.02 substitutions per nucleotide position and accession numbers are given in parenthesis.

RESULTS AND DISCUSSION

Solubilization index (SI)

Solubilization index based on colony diameter and halo zone diameter for bacterial isolates are shown in Figure 2. The isolated bacterial strains were capable of solubilizing tricalcium phosphate present in NBRIP medium and they formed large halos with varied intensity. It was seen that halo-zone increased with increase in colony diameter. Solubilization index was found to reached the peak at 2 to 4 days followed by gradual reductions in all the strains as incubation progressed. Among the isolates, the highest solubilization index was recorded from PSB-8 and PSB-13 (SI = 3.5) plates followed by PSB-1 and PSB-15 (SI = 3.25 to 3.00). The time taken for exhibiting the highest solubilization index was 2 and 3 days, respectively, for PSB-13 and PSB-1, whereas for the strains PSB-8 and PSB-15, the highest solubilization index was recorded at day 4 of the incubation. The lowest solubilization index (SI = 2.75) was found to be exhibited from PSB-5 at day 5 of the incubation.

Correlation coefficient values showed a significant ($P \leq 0.05$) positive correlation between phosphate solubilization and solubilization index. A significant negative correlation between pH and solubilization index was also shown among all the strains throughout the incubation. The results in this study were in agreement with that of Whitelaw (2000) and Alam et al. (2002) who also observed similar correlations.

Studies on agar plates revealed that the clear zones formed by phosphate solubilizing microorganisms are due to the production of organic acids into the surrounding medium as described by Gaur (1990). However, it has previously been reported that most of the phosphate solubilizing micro-organisms loss their ability to form halo-

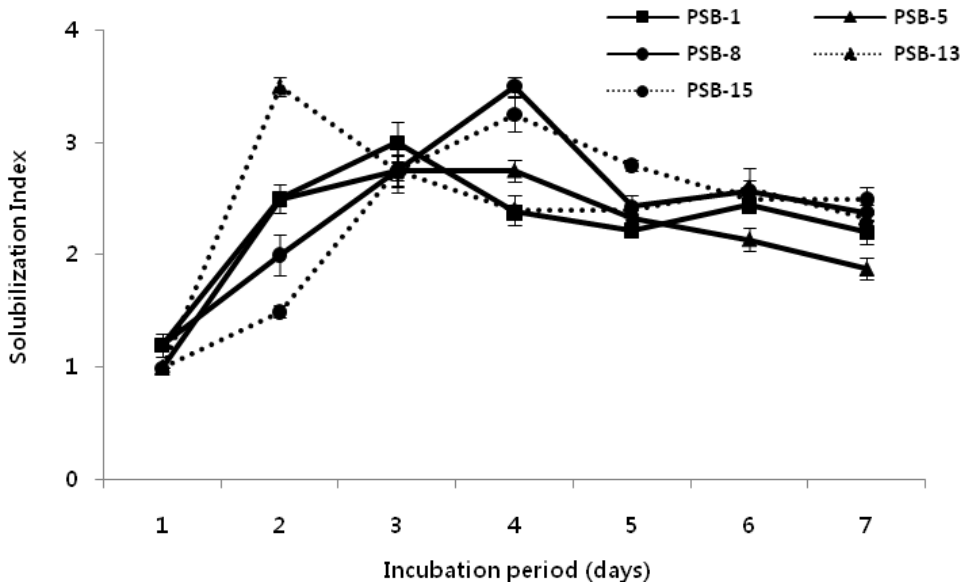

Figure 2. Solubilization index of isolated phosphate solubilizing bacterial strains on NBRIP plates. Values given are the means (n = 3) ± standard deviation.

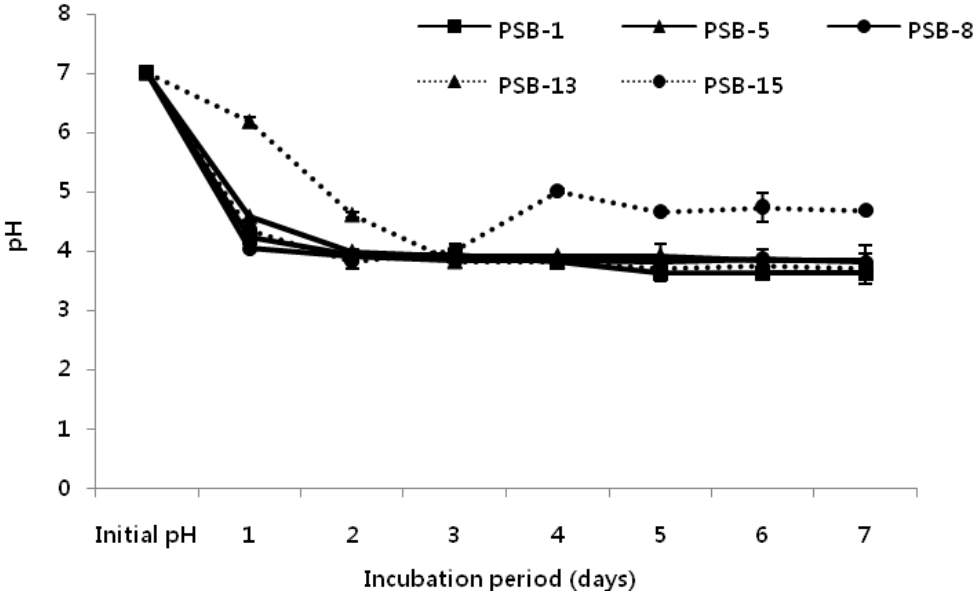

Figure 3. Changes of pH in NBRIP culture medium containing phosphate solubilizing bacterial strains. Values given are the means (n = 3) ± standard deviation.

zone on medium due to repeated sub-culturing (Alam et al., 2002). Generally, phosphate solubilizing microorganisms are routinely screened based on this solubilization index. However, the reliability of this halo-based technique is questioned as many isolates which did not produce any visible halo zone on agar plates could still solubilize various types of insoluble inorganic phosphates in liquid medium (Gupta et al., 1994).

Inorganic phosphate solubilizing ability

Periodic changes in the pH of NBRIP medium and soluble phosphorus content which has been released from the inorganic phosphorus during 7 days of incubation are presented in Figures 3 and 4 respectively.

The $Ca_3(PO_4)_2$ containing NBRIP medium inoculated with bacterial strains was turbid initially, however, turned

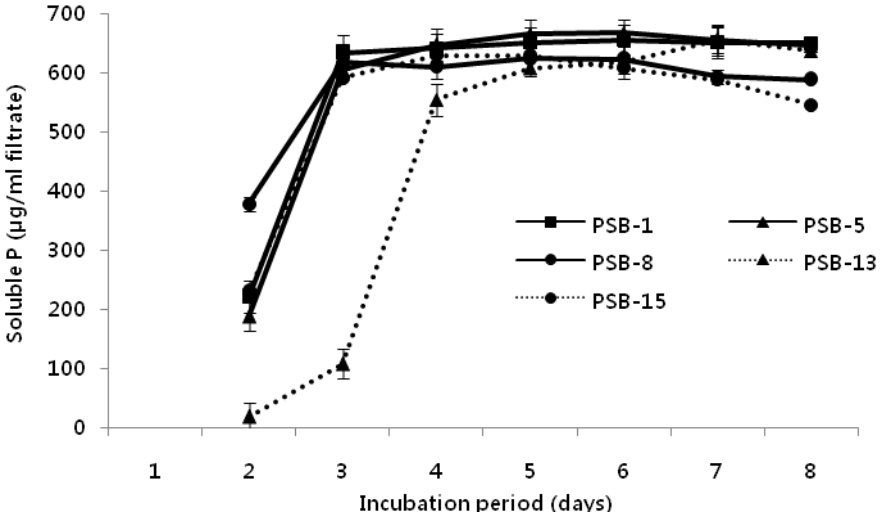

Figure 4. Insoluble phosphate solubilization (µg/ml of filtrate) of isolated phosphate solubilizing bacterial strains. Values given are the means (n = 3) ± standard deviation.

to clear within 2 days due to the utilization of inorganic phosphorus by inoculated strains. It was evident from the results that phosphate solubilizing bacterial isolates increased available phosphorus concentration in the medium and the bulk of the increment occurred during the first 2 to 3 days of the incubation. The bacterial isolates PSB-1, PSB-5 and PSB-8 solubilized significantly higher amount of inorganic phosphate within a day as compared to other bacterial strains (> 600 µg/ml of culture filtrate). Time course for solubilization is also a matter of concern due to the fact that the quantity of soluble phosphorous remained released as incubation progressed. However, subsequent drop in soluble phosphorus level was observed on later days of the incubation.

The decrease could be due to the availability of soluble phosphorus in the culture medium, which has an inhibitory effect on further phosphate solubilization (Varsha-Narsian et al., 1994). Moreover, formation of organo-phosphate compound was induced by organic metabolites released, which in turn, could reduce the amount of available phosphorus (Illmer and Schinner, 1995). Some researchers have suggested that it is due to the depletion of nutrients in the culture medium especially carbon source for the production of organic acids and microbial activity (Kang et al., 2002; Kim et al., 2005; Chaiharn and Lumyong, 2009). In the case of control, no significant change in the content of soluble phosphorus was observed throughout the incubation period.

All the studied phosphate solubilizing isolates lowered the pH of the NBRIP medium as compared to the control where it remained constant. The reduction of pH was rapid during the initial stages of incubation (first 2 days) followed by slower decrease. However, some fluctuations in pH were observed from stain PSB-8. The inverse relationship between pH and soluble phosphorus concentration observed from all the bacterial strains suggested that acidification of the medium could facilitate the inorganic phosphate solubilization.

The phosphate solubilization is generally determined by the microbial ability to produce and release organic acids through their carboxylic groups, that dissolve phosphatic materials and/or chelate cationic partners of the phosphorus ions, that is, PO_4^{3-} directly, releasing phosphorus into solution (He et al., 2002; Stevenson, 2005; Chen et al., 2006; Vyas et al., 2007). However, organic acids are not considered to be the sole factor responsible for phosphate solubilization by bacteria (Chen et al., 2005). The protons associated with extracellular polysaccharides secreted by the microbes are also responsible for dissolution of phosphate in NBRIP medium (Illmer et al., 1995).

A similar inverse relationship between pH and available phosphorus were reported by other researchers (Hwangbo et al., 2003; Rashid et al., 2004; Song et al., 2008; Yasmin and Bano, 2011; Yu et al., 2011). The fluctuations in pH by some stains may be due to the chelation of organic acids with calcium ions (Ca^{2+}) in the tri calcium phosphate. Except these strains, all the other strains showed strong negative correlation ($r = -0.923 \pm 0.1$, $P \leq 0.05$) between soluble phosphorus content and pH in the culture medium. The present results are also in agreement with other researchers who reported similar correlation (Hwangbo et al., 2003; Yu et al., 2011).

Organic acid production

Organic acids produced from isolated phosphate solubilizing microorganisms are presented in Table 1. Decrease in pH of broth coinciding with increase in phosphate solubilization by isolated strains indicated the production of organic acids. Gluconic acid was the key acid produced

Table 1. Organic acids produced by isolated phosphate solubilizing microorganisms. Values given here are the means (n = 3). ND– Not detectable.

Strain	Day 1	Day 2	Day 3	Day 4
Gluconic acid (µg/ml)				
PSB -1	2195	6277	10982	10392
PSB -5	921	7796	10987	10662
PSB -8	317	415	437	418
PSB -13	44	3901	10725	10331
PSB -15	38	9776	15686	15156
Oxalic acid (µg/ml)				
PSB -1	9.09	11.22	13.11	12.24
PSB -5	1.92	18.28	19.28	17.45
PSB -8	6.18	5.08	5.08	1.95
PSB -13	ND	ND	ND	ND
PSB -15	94.72	125.89	147.67	126.84
Citric acid (µg/ml)				
PSB -1	28.86	59.71	181.81	125.91
PSB -5	ND	ND	ND	ND
PSB -8	95.74	100.54	125.84	111.34
PSB -13	25.54	39.68	62.29	24.64
PSB -15	ND	ND	ND	ND

by all the strains followed by oxalic and citric acids. However, strain PSB-13 did not produce oxalic acid, while stains PSB-5, PSB-18 and PSF-1 did not produce citric acid. The highest amount of gluconic acid (12.47 mg/ml) and oxalic acid (0.121 mg/ml) was recorded to be produced by PSB-15 (Table 1). These results suggest that the ability of isolated strains to solubilize phosphate was attributed to their capacity to produce organic acids especially gluconic acid, oxalic acid and citric acid.

To understand the pattern of organic acid production, it is needed to analyze organic acids throughout the incubation period (Park et al., 2010). Therefore, we analyzed organic acid production at 24 h interval until day 4 of incubation. Results revealed that organic acid production in the medium increased with the incubation period, reaching the maximum on days 2 to 3 (Table 1). Soluble phosphorus concentration in the medium also increased during the same period of incubation. Therefore, it is apparent that the production of organic acid may enhance the phosphate solubilization.

Acid and alkaline phosphatase activities

The activity of acid and alkaline phosphatase varied greatly among the isolates. The strains PSB-5 showed the highest activity of acid phosphatase (80 U) and alkaline phosphatase (88 U) after 2 days of the incubation. In addition to PSB-5, the strain PSB-2 also showed high alkaline phosphatase activity (85 U) after 2 days of incubation (Figures 5 and 6). The strain PSB-15 showed the lowest activity of acid phosphatase (51 U) and alkaline phosphatase (62 U) as compared to the other strains. According to the results, it is apparent that the amount of phosphorus released depends upon the efficiency of the phosphatase activity of the strain of concern.

However, though increased initially, both the activity of acid and alkaline phosphatase was found to be decreased rapidly at the later stages of the incubation. As reported by Xiao et al. (2009), the reduction at the later stages could be attributed to the accumulation of soluble phosphorus, which inhibit the activity of acid and alkaline phosphatase.

Correlation of both acid and alkaline phosphatase activity to other studied parameters varied greatly from strain to strain. However, a strong positive correlation ($r = 0.91 \pm 0.2$, $P \leq 0.05$) between acid phosphatase and alkaline phosphatase activity could be observed for all the strains, except PSB-8.

It is clear from the results that the strains which demonstrated moderate to low phosphatase activity can release high amount of phosphorus by solubilizing inorganic phosphate. Therefore, demonstration of high phosphatase activity and releasing high amount of phosphorus and vice versa may be due to the specificity of the phosphatase (Deepa et al., 2010). Phosphatase activity contributes to the solubilization of organic phosphates in soil (Jakobsen et al., 2005). However, phosphatase does not act directly on inorganic phosphate solubilization, though phosphatase activity may participate in lowering the pH of the culture medium by the dephosphorylating action and the production of acids (Achal et al., 2007). These dephosphorylation reactions are involved in the hydrolysis of phosphoester or phosphoanhydride bonds. The phosphohydrolases are clustered in acid or alkaline conditions. The acid phosphohydrolases exhibit optimal catalytic activity at neutral and acidic medium than the alkaline phosphatases (Deepa et al., 2010). Moreover, they can be further classified as specific and non-specific acid phosphatase, in relation to their substrate specificity.

Conclusion

All the isolated bacterial strains were found to exhibit phosphate solubilizing capacity in varying degrees ranging from 624 to 667 µg/ml of culture filtrate. Solubilization index of these isolates was found to be within the range of 2.6 to 3.5. A strong negative correlation ($r = -0.923 \pm 0.1$, $P \leq 0.05$) was observed between the amount of phosphorus release and pH in the culture medium. The strains PSB-5 showed the highest activity of acid phosphatase (80 U) and alkaline phosphatase (88 U) as compared to the other strains. Acidification of the medium along with the phosphatase activity may be the key factors attributed to phosphate solubilization. Among the isolated bacterial strains, PSB-1 (*Pantoea agglomerans*), PSB-5 (*Burkholderia anthina*) and PSB-8 (*Enterobacter ludwigii*) solubilized significantly higher amount of inorganic phosphate within a day demonstrating their higher potential to be used as soil ino-

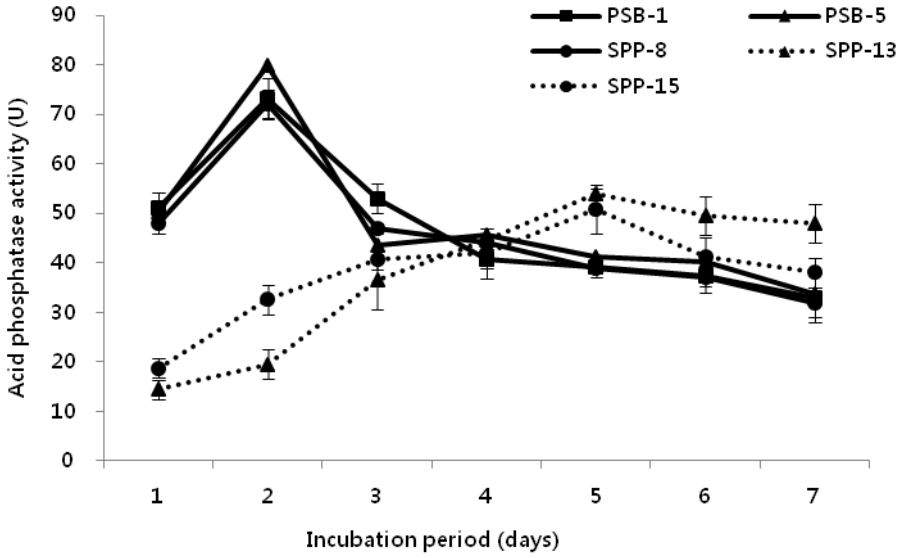

Figure 5. Acid phosphatase activity of isolated phosphate solubilizing bacterial strains. Values given are the means (n = 3) ± standard deviation.

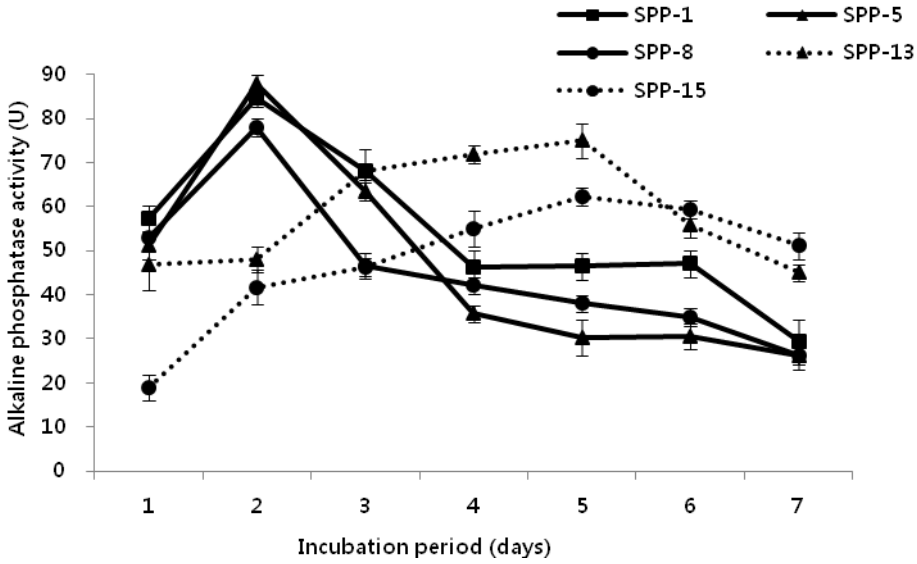

Figure 6. Alkaline phosphatase activity of isolated phosphate solubilizing bacterial strains. Values given are the means (n = 3) ± standard deviation.

inoculants to enhance soil fertility and plant growth. *In vivo* studies with the most efficient bacterial isolates would further confirm the present findings.

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