ISSN 0367-6315
Korean J. Soil Sci. Fert. 45(6), 998-1003 (2012)
http://dx.doi.org/10.7745/KJSSF.2012.45.6.998

Article

Influence of Different pH Conditions and Phosphate Sources on Phosphate Solubilization by *Pantoea agglomerans* DSM3493

Buddhi Charana Walpola, Mi-Jung Keum, and Min-Ho Yoon*

Department of Bio-Environmental Chemistry, College of Agriculture and Life Sciences, Chungnam National University, Daejeon, 305-764, Korea

***Pantoea agglomerans* DSM3493 was isolated from green house soils collected from Chungchugnam-do province, Gongju-Gun area in South Korea and phosphate solubilization and organic acid production of the strain were assessed using three types of insoluble phosphate sources (Ca phosphate, Fe phosphate and Al phosphate) under three different pH conditions (7, 8 and 9). The highest Ca phosphate solubilization (651 μg mL^{-1}) was recorded at pH 7 followed by pH 8 and 9 (428 and 424 μg mL^{-1} respectively). The solubilization rate was found to be 80.4, 98.1 and 88.7 μg mL^{-1} (for Fe phosphate containing medium) and 9.3, 12.1 and 29.8 μg mL^{-1} (for the Al phosphate containing medium) respectively at pH 7, 8 and 9. Though increasing pH of the medium caused reduction in the rate of solubilization of Ca phosphate, solubilization of Fe and Al phosphates enhanced with increasing pH. By contrast, the highest amount of organic acid was produced with Ca phosphate while the lowest was recorded with the presence of Al phosphate. Among the organic acids, gluconic acid production was found to be the highest, followed by oxalic acid and citric acid regardless the source of phosphate. Results can thus be concluded that the production of organic acids appears to play a significant role in the inorganic phosphate solubilization.**

Key words: Organic acids, Phosphate solubilization, *Pantoea agglomerans* DSM3493

Introduction

Phosphorous (P), one of the major essential macro-nutrients required by plants, is found in soil as inorganic and organic forms both of which are used as source of fertilizers. However, the majority of applied phosphatic fertilizers are readily fixed in soil and become unavailable to plants. Inorganic phosphates in acidic soils are associated with iron (Fe) and aluminum (Al) compounds; whereas calcium (Ca) phosphates are predominant inorganic phosphate form in neutral or calcareous soils (Gyaneshwar et al., 2002). Therefore, unfavorable pH along with high reactivity of aluminum and iron in soils decrease phosphorous availability as well as phosphatic fertilizer efficiency (Hao et al., 2002).

There are some microorganisms reported to be involved in solubilizing insoluble phosphate complexes, and they are called as phosphate solubilizing microorganisms (PSMs). The mechanism of phosphate solubilization by PSMs is associated with acidification, chelation, exchange reactions and release of low molecular weight organic acids such as gluconic acids, oxalic acids, citric acids, succinic acids etc. (Chaiharn and Lumyong, 2009; Gulati et al., 2010). However, the major microbiological means by which insoluble phosphate compounds are solubilized is through the production of organic acids, accompanied by acidification of the medium. Hydroxyl and carboxyl groups of these acids chelate the cations bound to phosphate, thereby converting it into soluble forms. However phosphate solubilization is a complex phenomenon, which depends on many factors such as nutritional, physiological and growth conditions of the medium. PSMs isolated from different soils have been used in evaluating their mineral phosphate solubilizing activity and organic acid production with various phosphate sources such as Ca phosphate, Fe phosphate and Al phosphates (Fankem et al., 2006). However, information on the effect of pH for organic acid production under different phosphate sources is scanty. Therefore, the present study was conducted to assess the influence of different pH and

Received : 2012. 9. 10 Accepted : 2012. 11. 22
*Corresponding author : Phone: +82428216733
E-mail: mhyoon@cnu.ac.kr

source of phosphate on phosphate solubilization by *Pantoea agglomerans* DSM3493 isolated from green house soils in Chungchugnam-do province, Gongju-Gun area in South Korea.

Materials and Methods

Isolation of bacterial strain Soils used in isolating bacterial strain were collected from Chungchugnam-do province, Gongju-Gun area in South Korea. Field moist soil was mixed with sterile 0.85% NaCl solution and shaken for 30 minutes. Serial dilutions were inoculated using NBRIP (National Botanical Research Institute Phosphorus) agar plates containing 10 g glucose, 5 g $Ca_3(PO_4)_2$, 5 g $MgCl_2.6H_2O$, 0.25 g $MgSO_4.7H_2O$, 0.2 g KCl, 0.1 g $(NH_4)_2SO_4$ in 1 L distilled water (Nautiyal, 1999). The plates were incubated for 5 days at 30℃. 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℃.

Strain identification The partial sequencing of 16S rRNA for the bacterial strain was done with the help of DNA sequencing service, SOLGENT, Daejeon, South Korea. 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 (Fig. 1) was constructed using CLUSTAL X program, which involved sequence alignment by neighbor joining method and maximum parsimony using the MEGA4 program. 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.

Assay of inorganic phosphate solubilizing ability Bacterial strain was grown in sterilized liquid NBRIP medium (20 mL) at 30℃ for 2 days with continuous shaking at 150 rpm. Bacterial suspension (1×10^8 CFU mL^{-1}) was then transferred into a 500 ml flask (n=3 per strain) containing sterilized liquid NBRIP medium (200 mL) and incubated for 7 days with continuous shaking at 30℃. Sterilized uninoculated medium served as a control. Aliquot (10 mL) of each culture and control was taken 2, 5 and 7 days after inoculation 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 glass electrode equipped pH meter. Phosphorus availability was determined using phospho-molybdate blue color method (Murphy and

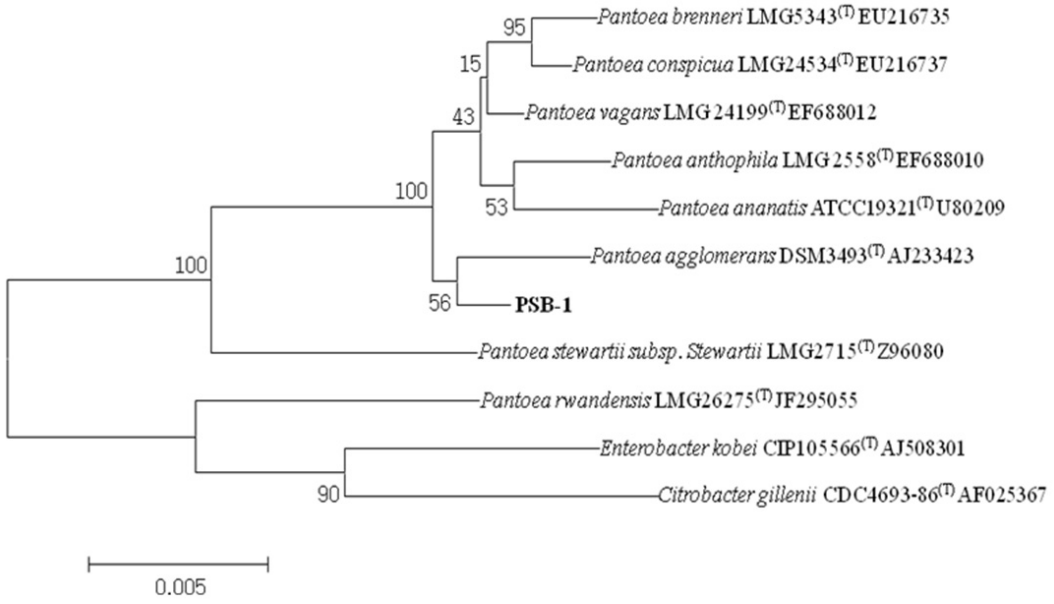

Fig. 1. Phylogenetic tree based on 16S rDNA gene sequences, showing the position of isolated efficient phosphate solubilizing bacterial strain with respect to related species. The scale bar indicates 0.005 substitutions per nucleotide position and accession numbers are given in parenthesis.

Riley, 1962).

The Ca phosphate solubilization assay was performed using NBRIP medium which contained $Ca_3(PO_4)_2$ while Al phosphate (NBRIP-AlP) and Fe phosphate (NBRIP-FeP) solubilization was assayed by adding 4 g L^{-1} $AlPO_4$ or 6 g L^{-1} $FePO_4.2H_2O$ instead of $Ca_3(PO_4)_2$ in NBRIP medium. These amounts are equal to the amount of phosphorus in the standard NBRIP medium. All the experiments were conducted under three different pH conditions (pH 7, 8 and 9). In all cases, phosphate solubilization and pH of the culture medium were measured as described earlier.

Assay of organic acid production To determine the organic acid composition of the different cultures, aliquots from the above 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 40^0C. Mobile phase consisted of 0.008 M H_2SO_4 run at a flow rate of 0.2 mL min^{-1}. 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 known three organic acids.

Values are given as means ± SD for triplicate samples. All the data were analyzed by analysis of variance or by regression analysis. Differences were considered to be significant at the $P \leq 0.05$ level.

Results and Discussion According to 16S rRNA sequence analysis, the strain was identified as *Pantoea agglomerans* DSM3493. Phylogenetic tree (Fig. 1) shows the position of isolated phosphate solubilizing bacterial strain with respect to related species.

Periodic changes in the soluble phosphorus content, which has been released from calcium phosphate (NBRIP-CaP) in the NBRIP medium under different pH conditions, during 7 days of incubation, are presented in Fig. 2a. Production of gluconic acid, oxalic acid and citric acid from the strain is represented in Fig. 2b, 2c and 2d respectively. As depicted Fig. 2a, the highest phosphate solubilization was recorded at pH 7 (651 µg mL^{-1}) followed pH 8 and 9 (428 and 424 µg mL^{-1} respectively). Though difference was not significant ($P \leq 0.05$), phosphate solubilization decreased by 34.3% and 34.9% respectively at pH 8 and 9.

Organic acid production in the culture medium increased as incubation progressed, reaching to the maximum at day 5 (Fig. 2b, 2c and 2d). As shown in Fig. 2b, 2c and 2d reduction in medium pH was parallel

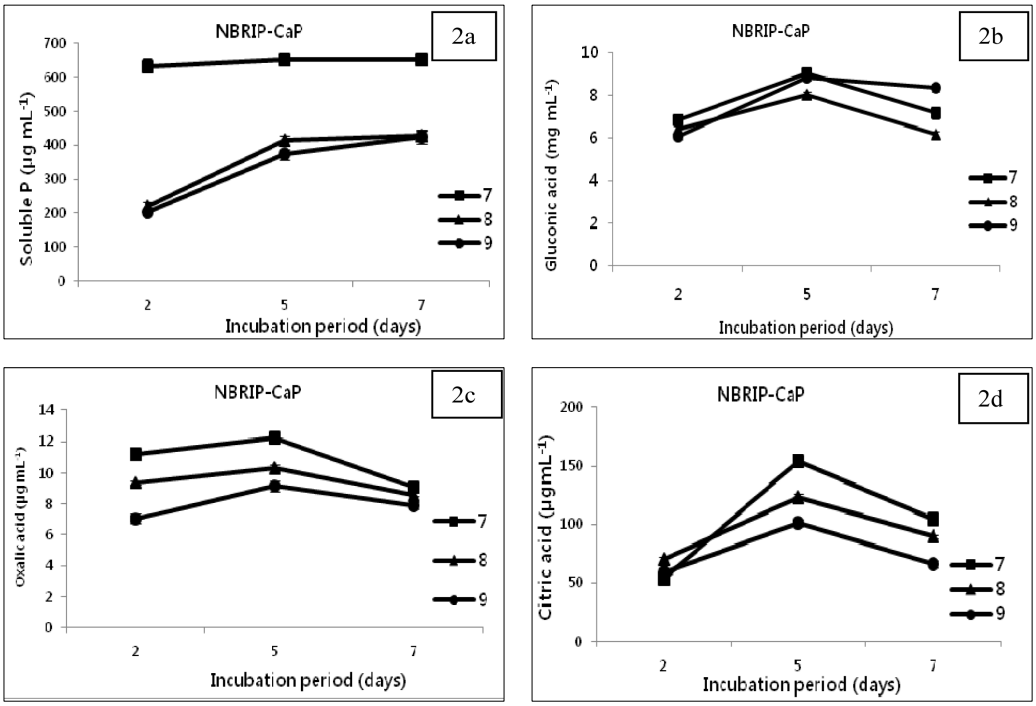

Fig. 2. Ca phosphate solubilization and organic acids production by *Pantoea agglomerans* DSM3493 under different pH conditions (a) Soluble phosphorous content (b) Gluconic acid production (c) Oxalic acid production (d) Citric acid production

to the increase of organic acids. At the end of the incubation period, the pH of the medium was found to be 3.63, 4.14 and 4.21 respectively from the initial pH 7, 8 and 9 (data not shown). Therefore, the production of organic acids played a significant role in acidification of culture medium, which in fact has contributed positively to the solubilization of inorganic phosphate. Gluconic acid (9.01-8.05 mg mL^{-1}) was the key organic acid produced by the *Pantoea agglomerans* followed by oxalic acid (12.24-9.13 µg mL^{-1}) and citric acid (154.33-101.25 µg mL^{-1}). However, remarkable decrease in the production of all three acids was observed with the increment of pH from 7 to 9.

Releasing pattern of soluble phosphorus into NBRIP medium, which contained Fe phosphate and Al phosphate (NBRIP-FeP and NBRIP-AlP) is shown in Fig. 3a and Fig. 4a; whereas the production of gluconic acid, oxalic acid and citric acid by the strain is shown in Fig. 3b, 3c, 3d and Fig. 4b, 4c, 4d respectively for Fe phosphate and Al phosphate.

As shown in graphs, Fe phosphate and Al phosphate solubilizing ability of *Pantoea agglomerans* DSM3493 was lower than that of the Ca phosphate, which is in consistent with the findings of Son et al. (2006), who observed low Fe phosphate (28 mg L^{-1}) and Al phosphate (19 mg L^{-1}) solubilization by *Pantoea agglomerans* R-42. This may be due to the adaptive nature of the enzyme that is responsible for solubilizing Ca phosphate (Banik and Dey, 1982). Panhwar et al. (2012) and Parasanna et al. (2011) have reported similar findings with isolated phosphate solubilizing microorganisms that solubilized Ca phosphate to a greater extent than rock phosphate, Al phosphate and Fe phosphate. Elaborating their findings, they have suggested that solubility of phosphorous might be associated with an activity of certain microbes in preferable phosphate sources or due to the activity of phosphatase enzyme. However, though solubilization of Fe phosphate and Al phosphate in the present study is comparatively lower than that of Ca phosphate, *Pantoea agglomerans* DSM3493 is shown to be capable of solubilizing hardly soluble Fe and Al phosphates to a certain extent (98.1 µg mL^{-1} and 29.8 µg mL^{-1} respectively for Fe phosphate and Al phosphate). Contrary to Ca phosphate solubilization, which recorded the highest phosphate solubilization at pH 7, the highest Fe phosphate and Al phosphate solubilization recorded at pH 8 and 9 respectively.

After 7 days of the incubation, the pH values in the cultures reduced to 3.63, 4.14 and 4.21 from the initial

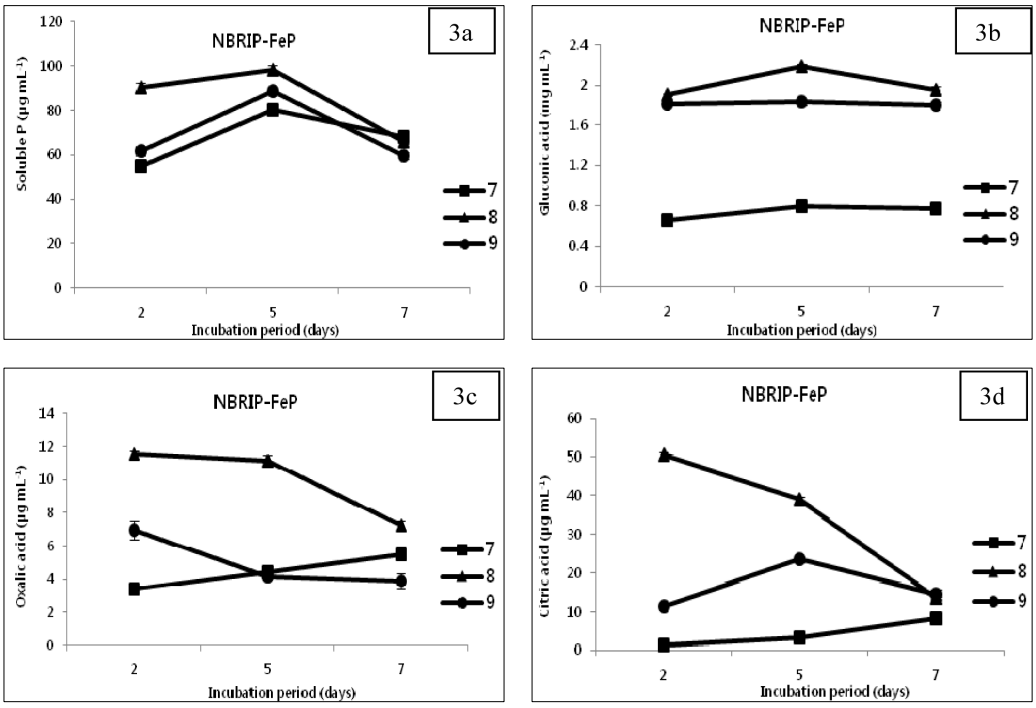

Fig. 3. Fe phosphate solubilization and organic acids production by *Pantoea agglomerans* DSM3493 under different pH conditions (a) Soluble phosphorous content (b) Gluconic acid production (c) Oxalic acid production (d) Citric acid production.

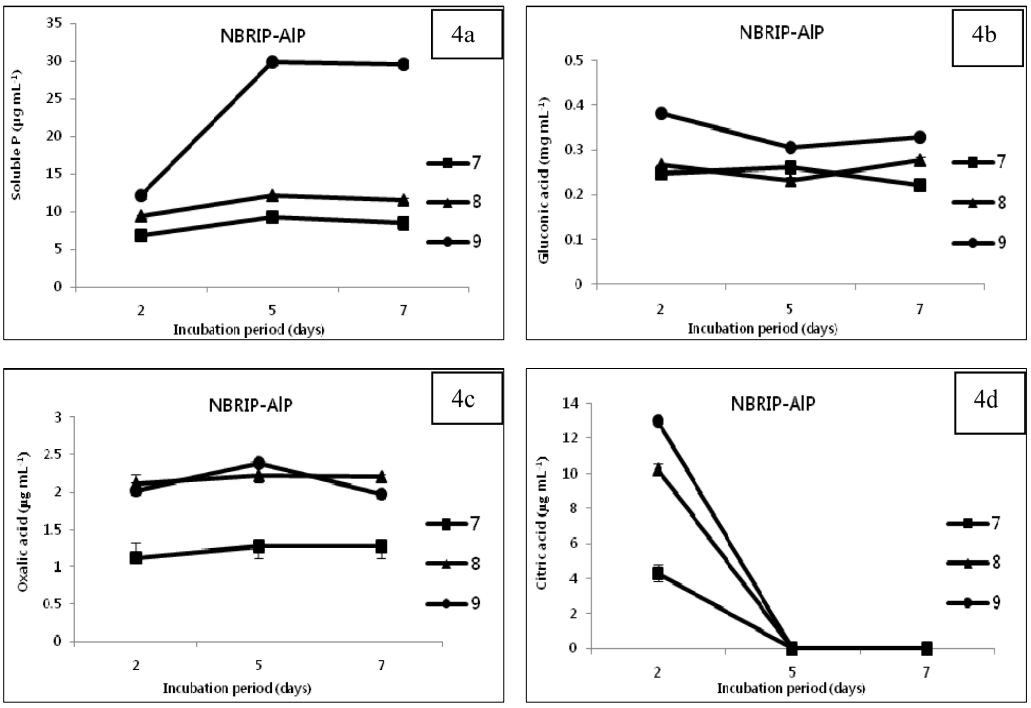

Fig. 4. Al phosphate solubilization and organic acids production by *Pantoea agglomerans* DSM3493 under different pH conditions (a) Soluble phosphorous content (b) Gluconic acid production (c) Oxalic acid production (d) Citric acid production

pH 7, 8 and 9 respectively (data not shown). In all cases, insoluble phosphate solubilization was accompanied by a distinct pH decrease. However, no positive correlation was found between pH and phosphate solubilization in the presence of Fe phosphate and Al phosphate. This is in agreement with the results reported by Son et al. (2006) and Narsian and Patel (2000), who too had not observed a correlation between pH and the amount of soluble phosphorus released with Fe phosphate and Al phosphate as sole phosphate source.

However, a few reports is available on effective soil microorganisms in solubilizing hardly soluble Fe or Al phosphates (Antoun, 2002; Barroso and Nahas, 2006). *Penicillum rugulosum* was reported to be more efficient solubilizer for Al phosphate and Fe phosphate than hydroxyapatite (Reyes et al., 1999). Fungal strain *Fomitopsis* sp. PS102 was also capable of solubilizing $AlPO_4$, but was almost unable to solubilize hydroxyapatite (Kang et al., 2002).

As shown in Fig. 3b, 3c, 3d and 4b, 4c, 4d, organic acids production was also found to be very low when Fe phosphate and Al phosphate used as a sole phosphate source. By contrast, the highest amount of organic acid production was found in the presence of Ca phosphate while the lowest recorded with the Al phosphate. Among the organic acids, production of gluconic acid was found to be the highest, followed by oxalic acid and citric acid regardless the source of phosphate.

According to the results, organic acid production was greatly varied with the medium pH in the presence of Fe phosphate and Al phosphate. The highest amount of gluconic acid (2.19 mg L^{-1}), oxalic acid (11.51 µg mL^{-1}) and citric acid (50.67 µg mL^{-1}) production was recorded at pH 8 followed by pH 9 in the presence Fe phosphate while the highest phosphate solubilization was recorded at pH 8 (98.1 µg mL^{-1}) followed by pH 9 (88.7 µg mL^{-1}). Similar to these findings, the highest gluconic acid (0.38 mgL^{-1}), oxalic acid (2.21 µg mL^{-1}) and citric acid (12.96 µg mL^{-1}) production with Al phosphate was recorded at pH 9 followed by pH 8. Based on the results, a positive correlation could be observed between organic acid production and phosphate solubilization at different pH conditions. Therefore, it is apparent that the production of organic acids plays a significant role in inorganic phosphate solubilization.

Corresponding to these results, Fankem et al. (2006) reported that the type and concentration of organic acid relatively vary from strain to strain as well as appear to be independent from the source of phosphate. They also observed the highest amount of organic acid production

with Ca phosphate and a little amount of organic acid production with Al phosphate.

Conclusion

Though the isolated strain *Pantoea agglomerans* DSM3493 solubilized Ca phosphate to a greater extent, its solubilizing ability of Al phosphate was very low. Ca phosphate solubilization decreased with increasing pH; whereas Fe and Al phosphate solubilization increased with increasing pH. A positive correlation was found between phosphate solubilization and organic acid production. Therefore, the production of organic acids was appeared to play a significant role in inorganic phosphate solubilization.

References

Antoun, H. 2002. Field and greenhouse trials performed with phosphate-solubilizing bacteria and fungi. In: First International meeting on microbial phosphate solubilization. Salamanca, Spain, 16-19 July, pp. 29-31.

Banik, S. and B. K. Dey. 1983. Phosphate solubilizing potentiability of the microorganisms capable of utilizing aluminium phosphate as a sole phosphate source. Zentralbl Mikrbiol. 138:17-23.

Barroso, C. V., G. T. Pereira, and E. Nahas. 2006. Solubilization of $CaHPO_4$ and $AlPO_4$ by Aspergillus niger in culture media with different carbon and nitrogen sources. Braz. J. Microbiol. 37: 434-438.

Chaiharn, M. and S. Lumyong. 2009. Phosphate solubilization potential and stress tolerance of rhizobacteria from rice soil in Nothern Thailand. World. J. Microbiol. Biotechnol. 25: 305-314.

Fankem, H., D. Nwaga, A. Deubel, L. Dieng, W. Merbach, and F. X. Etoa. 2006. Occurrence and functioning of phosphate solubilizing microorganisms from oil palm tree (*Elaeis guineensis*) rhizosphere in Cameroon. Afr. J. Biotech. 5**:** 2450-2460.

Gulati, A., N. Sharma, P. Vyas, S. Sood, P. Rahi, V. Pathania, and R. Prasad. 2010. Organic acid production and plant growth promotion as a function of phosphate solubilization by *Acinetobacter rhizosphaerae* strain BIHB 723 isolated from the cold deserts of the trans-Himalayas. Arch. Microbiol. 192: 975–983.

Gyaneshwar, P., K. G. Naresh, L. J. Parekh, and P. S. Poole. 2002. Role of soil microorganisms in improving P nutrition of plants. Plant Soil. 245: 83–93.

Hao, X., C. M. Cho, G. J. Racz, and C. Chang. 2002. Chemical retardation of phosphate diffusion in an acid soil as affected by liming. Nutr. Cyc. Agroecosyst. 64**:** 213–224.

Kang, S. C, C. G. Ha, T. G. Lee, and D. K. Maheswari. 2002 Solubilization of insoluble inorganic phosphates by a soil-inhabiting fungus *Fomitopsis sp*. PS 102. Curr. Sci. 82: 439-442.

Murphy, J. and J. P. Riley. 1962. A modified single solution method for the determination of phosphate in mineral waters. Anal. Chim. Acta. 27:31-36.

Narsian, V. and H. H. Patel. 2000. *Aspergillus aculeatus* as a rock phosphate solubilizer. Soil Biol. Biochem. 32: 559-565.

Nautiyal, C. S. 1999. An efficient microbiological growth medium for screening of phosphate solubilizing microorganisms. FEMS Microbiol. Lett. 170: 265-270.

Panhwar, Q. A., R. Othman, Z. A. Rahman, S. Meon, and M. R. Ismail. 2012. Isolation and characterization of phosphate solubilizing bacteria from aerobic rice. Afr. J. Biotech. 11**:** 2711-2719.

Prasanna, A., V. Deepa, P. Balakrishna Murthy, M. Deecaraman, R. Sridhar, and P. Dhandapani. 2011. Insoluble phosphate solubilization by bacterial strains isolated from rice rhizosphere soils from Southern India. Int. J. Soil Sci. 6:134-141.

Reyes, I., L. Bernier, R. R. Simard, P. Tanguay, and H. Antoun. 1999. Characteristics of phosphate solubilization by an isolate of a tropical *penicillium rugulosum* and two UV-induced mutants. FEMS Microbiol. Ecol. 28:291-295.

Son, H. J., G. T. Park, M. S. Cha, M. S. Heo. 2006. Solubilization of insoluble inorganic phosphates by a novel salt and pH tolerant *Pantoea agglomerans* R-42 isolated from soybean rhizosphere. Bioresour. Technol. 97: 204-210.