ISSN 2630-0540
Journal of Technology and Innovation in Tertiary Education, Vol.2, No.1, pp. 27-33, January-June 2019

doi:

Screening of Phosphate Solubilizing Bacteria from Rhizosphere of Rubber Tree (Hevea brasiliensis Muell Arg)

Thanakorn Saengsanga
Tarntip Rattana
Sutat Termsaithong
Environmental Science Program, Faculty of Science and Technology
Nakhon Ratchasima Rajabhat University, Nakhon Ratchasima, Thailand
Email: thanakorn.s@nrru.ac.th

Abstract

The preliminary study aimed at screening the phosphate solubilizing bacteria (PSB) from root rhizosphere of rubber trees (Hevea brasiliensis Muell Arg) in Nongki district, Buriram province, Thailand on Pikovskaya's agar medium, specific culture medium. Obtained PSB were then examined for their extracellular enzyme production such as amylase, cellulase, lipase and protease by plate assays. The results revealed that seven isolates of PSB were retrieved from rhizosphere of rubber trees. The phosphate solubilization index was examined by using tri-calcium phosphate (TCP) as sole phosphorus source. Solubilization index by the isolates were observed in ranging from 2.28 to 2.88, which were determined in PVK medium supplemented with 0.5% (w/v) of TCP after incubation at 30°C for 5 days. The results also showed that the highest SI (2.88) was obtained from strain NRRU-S5 followed by NRRU-T18 (2.86), NRRU-S16 (2.78) and the lowest of SI was observed by strain NRRU-S17 (2.28), respectively. Furthermore, all of the PSB strains were qualitative estimated in liquid PVK medium supplemented with 0.5% (w/v) of TCP. Phosphate solubilization by the strains were ranged from 35.8 to 192.5 µg/mL by Vanado-molybdate-yellow color method. It was confirmed that the NRRU-T18 was the best phosphate solubilizer by releasing soluble phosphate at the highest level of 192.5 µg/mL. The NRRU-S5, NRRU-S19, and NRRU-S16 were solubilized 168.8, 162.5 and 103.3 µg/mL, respectively. The lowest (35.8 µg/mL) phosphate solubilization was found in NRRU-S17. Finally, the enzymatic productions by PSB were analyzed. The results pointed to NRRU-S17 being able to produce amylase and protease while NRRU-T18 and NRRU-T12 being able to produce cellulase and amylase, respectively.

Keywords: *Phosphate solubilizing bacteria, rubber trees, rhizosphere*

1. Introduction

Phosphorus (P) is the second most important crop nutrient after nitrogen for plant growth and development. It is considered the limiting nutrient for crops in Thailand because most of soil phosphorus is often presented in the form of insoluble phosphates, leading to P-deficient in plants (Khan, Zaidi & Ahmad, 2014). To increase availability of phosphorus for plants, fertilizers are applied to soil. As known, large amount of P-fertilizers after application is rapidly transformed into insoluble form and adsorbed by Fe and Al oxides through legend exchange (Omar, 1998). In addition, insoluble phosphate can be dissolved into soluble phosphate by soil microorganisms as reported previously in pot experiments and under various field conditions (Pradhan & Sukla, 2005; Lavakush et al., 2014).

Root rhizosphere bacteria increasing useful phosphorus is known as phosphate solubilizing bacteria (PSB). It provides P availability to plants via mechanisms of organic acids production, chelation, exchange reactions, and polymeric substances formation (Chakkaravarthy et al., 2010), to mobilize insoluble phosphate from tri-calcium phosphate (TCP) in soil. Many PSB strains, Pseudomonas spp. (Lavakush et al., 2014) Baccillus spp. (Swain & Ray, 2009) Pantoea sp. and Enterobacter sp. (Park et al., 2011) were isolated and studied. Applications of PSB in agricultural practices have been reported in improving plant growth and productivity (Lavakush et al., 2014).

Rubber trees (Hevea brasiliensis Muell Arg) are an important economic plant for natural rubber production. P deficiency could affect rubber tree productivity seriously because it is an essential macronutrient. The use of microbial inoculants possessing phosphate solubilizing activity in agricultural soils is considered as an environmental-friendly alternative to further applications of chemical-based P fertilizers. Therefore, the aim of this study was to screen the PSB for their ability to solubilize tricalcium phosphate and their enzyme productions.

2. Materials and Methods

2.1 Isolation of phosphate solubilizing bacteria

To isolate phosphate solubilizing bacteria, rhizospheric soils of rubber trees were collected from mature plantations at Nongki district, Burirum province, Thailand. The samples were taken to the laboratory in polythene bags surface sterilized with 70% (v/v) ethanol. Ten grams of the rhizospheric soil sample was mixed with 90 ml of sterile normal saline (0.85% (w/v) NaCl) in 250 mL of Erlenmeyer flask. A 10-fold serial dilution was done and 0.1 mL aliquots (10^{-3} to 10^{-5}) were spread on Pikovskaya's agar medium (ingredients per liter; 10 g glucose, 0.5 g yeast extract, 0.1 g $MgSO_4.2H_2O$, 0.2 g NaCl, 0.2 g KCl, 0.002 g $MnSO_4.2H_2O$, 0.002 g $FeSO_4.7H2O$, 5 g $Ca_3(PO_4)_2$ (TCP) and 15 g agar, pH 7.0) (Pikovskaya, 1948). After incubation at 30°C for 3-5 days, the colonies exhibiting a clear zone (halozone) formed around colonies were considered phosphate solubilizing bacteria (PSB) and subject to determine their phosphate solubilization activity.

Obtained bacteria were confirmed for phosphate solubilization activity by an agar plate method. To prepare a starter, bacterial isolates were cultured in 5 mL of Nutrient broth (NB) at 30°C for 24 h. Bacterial suspension (10 µL) was dropped on Pikovskaya's agar medium and incubated at 30°C for 5 days. The appearance of the clear zone formed around colonies and the colony diameter was measured, calculated and expressed as a solubilization index as follow (Edi-Premona et al., 1996):

$$\text{Solubilization Index (SI)} = \frac{\text{Colony diameter + Halo zone diameter}}{\text{Colony diameter}}$$

2.2 Quantitative estimation of phosphate solubilization activity

The PSB were then analyzed for their ability to solubilize insoluble phosphate, TCP in liquid medium. The overnight of bacterial culture was inoculated in Pikovskaya's broth containing 0.5% (w/v) TCP and incubated at 30°C for 5 days. Simultaneously, un-inoculated control was also done under a similar condition. The cultures were harvested by centrifugation at 8000 rpm for 10 min. The soluble phosphate in supernatant was estimated by Vanado- molybdate-yellow color method as described by Kumer et al. (Kumar et al., 2012). The amount of soluble phosphate was determined by comparison with the standard curve. The pH of culture supernatants were also measured using a pH Meter.

2.3 Screening for enzyme production

2.3.1 Amylase

The bacteria isolates were spot-plated on a starch agar medium containing (ingredients per liter) 3 g beef extract, 10 g soluble starch and 15 g agar. After incubation, the plates were flooded with Gram's iodine (2.0 g KI and 1.0 g iodine in 300 ml distilled water) and observed for the clear zone of hydrolysis surrounding the colony.

2.3.2 Cellulase

The bacteria isolates were spot-plated on carboxy methyl cellulose (CMC) gar; ingredients per liter, 2 g $NH_4H_2PO_4$, 0.6 g KH_2PO_4, 0.4 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, 1.0 g yeast extract, 5.0 g CMC), and the plates were incubated at 30°C for 24 h. The plates were flooded with Gram's iodine for 3 to 5 min. Then, the plates were observed for the zone of clearance around the colony (Kasana et al., 2008)[11].

2.3.3 Lipase

The bacteria isolates were spot-plated on NA medium amended with tributyrin (1% v/v) and incubated at 30°C for 24 h. The formation of the clear zone surrounding the colony was considered a lipase producer.

2.3.4 Protease

The bacterial isolates were dropped on Skim milk agar (Himedia) and incubated at 30°C for 24 h. After incubation, the plates were observed for the clear zone surrounding the colony.

3. Results and Discussion

3.1 Isolation of phosphate solubilizing bacteria

The screening strategy used under rhizospheric soil of rubber tree plantation accomplished the identification of PSB colonies on PVK medium containing TCP as the sole P source. Seven colonies exhibiting a clear halo were observed on agar plates supplemented with 0.5% (w/v) TCP, indicating phosphate-solubilizing ability of the strains (Figure 1).

Figure 1: Solubilization of Tricalcium Phosphate on Pikovskaya by the Strain

To confirm this observation, all 7 purified isolates were tested for SI by the method of Edi-Premono et al. (1996). Table 1 reveals TCP solubilization by bacterial isolates in an agar plate assay. The results showed the zone of solubilization around the bacterial colonies (halo diameter) ranging from 7.7-11.8 mm, and colony diameter varying from 6.0-8.3 mm. The zone formation around the growing colony indicated a phosphate solubilizing activity. Of these results, the isolate NRRU-S5, -T18 and -S16 revealed the high phosphate solubilizing activity with solubilization index (SI) of 2.88, 2.86 and 2.78, respectively (see Table 1). The results also indicated that these bacteria were able to solubilize insoluble TCP. According to Tripti & Anshumali (2012), SI of PSB ranging from 1.4-3.0 was recorded. The zone formation could stem from their production of any organic acids such as citric acid, lactic acid, gluconic acid, succinic acid, and propionic acid (Chen et al., 2006).

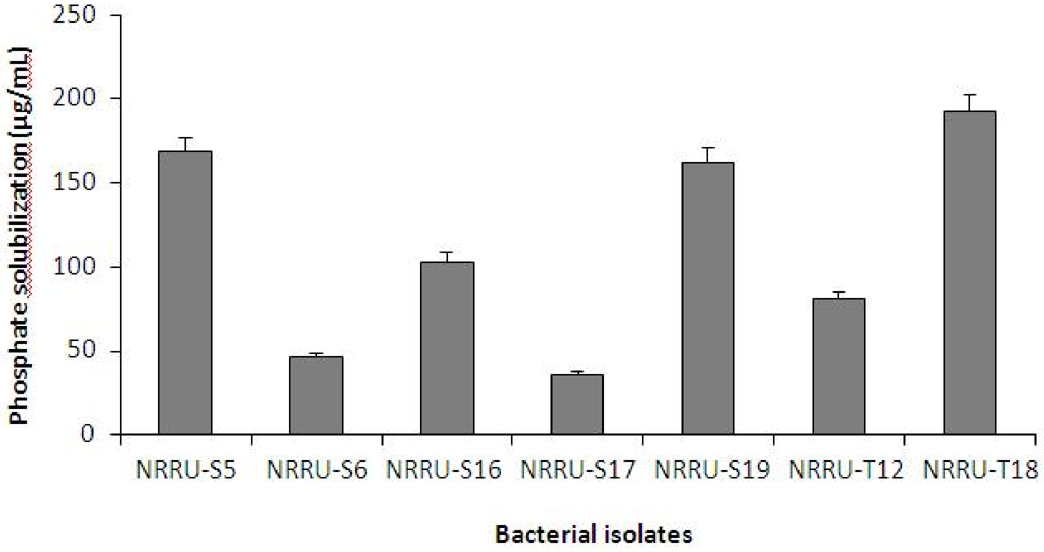
Table 1: Tri-calcium Phosphate Solubilization by Bacterial Isolates in Agar Assay

Bacterial Isolate	Colony diameter (mm)	Halo diameter (mm)	SI
NRRU-S5	6.3	11.8	2.88
NRRU-S6	6.8	10.5	2.56
NRRU-S16	6.8	12.0	2.78
NRRU-S17	6.0	7.7	2.28
NRRU-S19	6.8	9.5	2.41
NRRU-T12	8.3	11.3	2.36
NRRU-T18	7.0	13.0	2.86

3.2 Qualitative estimation of phosphate solubilization activity

The *qualitative estimation* of phosphate solubilization activity in liquid medium was performed in PVK medium supplemented with 0.5% (w/v) TCP. It was observed that phosphate solubilization by the strains was ranged from 35. 8 to 192. 5 µg/ mL by Vanado- molybdate- yellow color method. The highest phosphate solubilization 192. 5 µg/ mL was observed by NRRU- T18. The NRRU- S5, NRRU- S19, and NRRU- S16 were solubilized 168. 8, 162. 5 and 103. 3 µg/ mL, respectively. The lowest (35. 8 µg/ mL) phosphate solubilization was found in NRRU- S17. It should be noted that four strains showed phosphate solubilization activity above 100 µg/ mL (see Figure 2). The phosphate solubilization ability of the microorganisms depends on the insoluble P substrate in the culture medium and final pH (Gupta, Sabat & Parida, 2007). For example, *Gordonia* sp. CC-BC07 was capable of solubilizing 31.5 µg/mL in the medium initial pH 6.8-7.0 containing 0.5% (w/v) of TCP as a sole phosphorus source with final pH of 6.0 after cultivation (Chen et al., 2006) whereas *Pantoea agglomerans* R- 42 showed a high level of phosphate solubilization of 1367, hydroxyapatite 1357 and 1312 µg/ mL from dicalcium phosphate, hydroxyapatite and TCP, respectively (Singh & Hayashi, 1995). The pH of the culture was all dropped. NRRU- T18 showed the best P solubilization and it pH culture medium was dropped from 7.0 to 5.4 (data not shown). The potential mechanism for phosphate solubilization involves bacterial acidification via secretion of organic acids and the result in lowering pH. Even though the acid phosphatase activity is unlikely to influence over mineral P solubilization directly, it may participate in lowering pH-- indirectly contributing to mineral phosphate solubilization (Park et al., 2011).

Figure 2: Qualitative Estimation of Tricalcium Phosphate Solubilization by Bacterial Isolates



3.3 Screening for extracellular enzyme productions

Bacteria isolates were analyzed for enzyme productions (amylase, cellulase, lipase and protease) by the plate method. Besides phosphate solubilization activity, some isolates also showed other beneficial effects. NRRU- S17 produced amylase and protease by hydrolysis starch and skim milk. Similarly, NRRU-T12 and -T18 showed a positive for amylase and cellulase activity, respectively. Likewise, all seven isolates showed a negative lipase activity on tributyrin agar (see Table 2). *Phosphate solubilizer strains* as producers of cellulase are of interest to researchers from the biotechnological point of view (Singh & Hayashi, 1995) and in relation to the decomposition of agricultural residues remaining in the field after the crops are harvested (Swain & Ray, 2009).

Table 2: Enzyme Production by Bacterial Isolates

Bacteria isolate	**Enzyme production**			
	Amylase	**Cellulase**	**Lipase**	**Protease**
NRRU-S5	-	-	-	-
NRRU-S6	-	-	-	-
NRRU-S16	-	-	-	-
NRRU-S17	+	-	-	+
NRRU-S19	-	-	-	-
NRRU-T12	+	-	-	-
NRRU-T18	-	+	-	-

The sign (+) indicates the positive screening test and (-) indicates a negative screening test.

4. Conclusion

The endemic PSB isolated from rubber tree plantation rhizospheric soil at Buriram Province seems to have the capacity to solubilize insoluble forms of TCP. The PSB isolates NRRU-T18 exhibit the highest phosphate solubilization in vitro conditions. Furthermore, it can produce cellulase for degrading organic matters. This study provides a preliminary step of using the PSB strains from rhizosphere of rubber tree plantation for the development of effective microorganisms as bio-fertilizers for improving phosphorus plant nutrition. Further investigation should be pursued by characterizing other beneficial traits in promoting growth of plants.

5. The Authors

Thanakorn Saengsanga, Tarntip Rattana, and Sutat Termsaithong are staff members of the Environmental Science Program, Faculty of Science and Technology, Nakhon Ratchasima Rajabhat University, Thailand. These three researchers have their keen interest in screening phosphate solubilizing bacteria from rhizosphere of rubber trees (Hevea brasiliensis Muell Arg) and areas related to bacterial solubilization.

6. References

Chakkaravarthy, V. M. , Arunachalam. R. , Vincent. S. , Paulkumar. K. , G. Annadurai. (2010). Biodegradation of tricalcium phosphate by phosphate solubilizing bacteria. *Journal of Biological Sciences, 10*(6), 531-535.

Chen, Y.P., Rekha, P.D., Arun, A.B., Shen, F.T., Lai, W.A., Young, C.C. (2006). Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities. *Applied Soil Ecology, 34*, 33-41.

Edi-Premono, Moawad, M.A., Vleck, P.L.G. (1996). Effect of phosphate solubilizing Pseudomonas putida on the growth of maize and its survival in the rhizosphere. *Indonesian Journal of Crop Sciences, 11*, 13-23.

Gupta N., Sabat, J., Parida, R. (2007). Solubilization of tricalcium phosphate and rock phosphate by microbes isolated from chromite, iron and manganese mines. *Acta Botanica Croatica, 66*(2), 197-204.

Kasana, R. C. , Salwan, R. , Dhar, H. , Dutt, S. , Gulati, A. (2008). A rapid and easy method for the detection of microbial vellulases on agar plates using Gram's iodine. *Current Microbiology, 57*, 503-507.

Khan, M.S., Zaidi, A., Ahmad, E. (2014). Mechanism of Phosphate Solubilization and Physiological Functions of Phosphate- Solubilizing Microorganisms. In Khan, M.S., Zaidi, A., Musarrat, J. (Eds). (2014). *Phosphate Solubilizing Microorganisms: Principle and Application of Microphos Technology*. Springer Cham Heidelberg, 31-62.

Kumar, A. , Kumar, A. , Devi, S. , Patil, S. , Payal, C. , Negi, S. (2012). Isolation, screening and characterization of bacteria from rhizospheric soils for different plant growth promotion (PGP) activities: an in vitro study. *Recent Research in Science and Technology, 4*(1), 1-5.

Lavakush, Yadav J., Verma, J.P. Jaiswal D.K., Kumar. A. (2014). Evaluation of PGPR and different concentration of phosphorus level on plant growth, yield and nutrient content of rice (Oryza sativa). *Ecological Engineering, 62*, 123-128.

Oliveira, C.A., Alves, V.M.C., Marriel, I.E. Gomes, E.A., Scotti, M.R., Carneiro, N.P., Guimarães, C. T. , Schaffert R. E. , Sá, N. M. H. (2009). Phosphate solubilizing microorganisms isolated from rhizosphere of maize cultivated in an oxisol of the Brazilian Cerrado Biome. *Soil Biology and Biochemistry, 41*(9), 1782-1787.

Omar, S. A. (1998). The role of rock- phosphate- solubilizing fungi and vesicular- arbusular-mycorrhiza (VAM) in growth of wheat plants fertilized with rock phosphate. *World Journal of Microbiology and Biotechnology, 14,* 211-218.

Park, J. H. , Bolan, N. , Megharaj, M. , Naidu, R. (2011). Isolation of phosphate solubilizing bacteria and their potential for lead immobilization in soil. *Journal of Hazardous Materials, 185*(2- 3), 829-386.

Pikovskaya, R. I. (1948). Mobilization of phosphorus in soil connection with the vital activity of some microbial species. *Microbiology, 17,* 362-370.

Pradhan, N. , Sukla, L. B. (2005). Solubilization of inorganic phosphates by fungi isolated from agriculture soil. *African Journal of Biotechnology, 5*(10), 850-854.

Singh, A. and Hayashi, K. (1995). Microbial cellulases: protein architecture, molecular properties and biosynthesis. *Advances in Applied Microbiology*, *40,* 1-44.