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Isolation and screening phosphoric solubilizing bacteria from organic anthill fertilizer and phosphorus release capacity

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Abstract

Phosphate solubilizing bacteria (PSB) play role in solubilizatiion and mineralization of inorganic phosphate in soil to soluble organic phosphate (P). The organic P is available to plant growth. It is also a major microorganism in P biofertilizer. This work was to investigate the occurrence of PSB from liquid organic anthill fertilizer and their capacity for releasing soluble phosphate. Population of total bacteria and PSB was estimated by using plate count method. The result indicated that the population of total bacteria and PSB ranged from 240 x 10^5 – 950 x 10^5 CFU g^{-1} and 0.32 x 10^5 – 16.0 x 10^5 CFU g^{-1} soil on plate count agar and Pikovskaya (PVK) agar medium, respectively. Twenty five PSB showed diversity of different colony morphology on PVK agar medium. Preliminary test on efficiency of soluble phosphate production observed from hydrolytic capacity (HC) calculating from ration of diameter of clear zone and colony. Their HC ranged 1 – 4.60. Five PSB isolates such as 2-2A, 2-7, 3-5, 8-2A and 8-4 were selected as high efficiency of PSB from value of HC at 4.60, 3.40, 3.40, 3 and 2.90 respectively. They were tested releasing soluble phosphate in PVK broth supplement with $FePO_4$, $AlPO_4$, and $Ca_3(PO_4)_2$. The result revealed that 2-2A isolates plays highest of soluble phosphate concentration in broth supplement with $FePO_4$, $AlPO_4$, and $Ca_3(PO_4)_2$ at 4.80, 6.30 and 8.20 mg mL^{-1} respectively.

Keywords: Screening; Phosphate solubilizing bacteria; Phasphate fertilizer; Anthill

1. Introduction

Phosphorus (P) is the second essential macronutrient after nitrogen (N) for the overall plant growth and productivity [1, 2]. It plays an important role in plants for physiological activities including development of good root system and utilization of carbohydrate [3, 4]. Moreover, it influences to the key metabolic processes of the

vital cellular activities such as cell division, development, breakdown of sugar, energy transport (ATP, ADP), transport within the plant, signal transduction for genetic characteristics, macromolecular biosynthesis, photosynthesis and respiration of plants [5, 6]. Phosphorous deficiency in plant results in the leaves turning brown accompanied by small leaves, weak stem and slow development. However, naturally soils are source of insoluble organic and inorganic phosphates for plant requirement [7, 8].

Phosphorus enters in soils as inorganic phosphate from parent rock and as organic phosphate from decayed plant, animal, and microorganisms including animal manures. Dynamics of phosphorus in soil is characterized by physicochemical processes as sorption-desorption and biological processes as immobilization-mineralization. Phosphate anions (PO_4^-) can be immobilized into insoluble forms by precipitation with metal cations in acidic soil such as Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+} [7, 9]. The complex forms of insoluble phosphate are $Ca_3(PO_4)_2$, $FePO_4$, $AlPO_4$. These forms are not efficiently taken up by the plants. Some studies reported that soils contain very little total P of only 0.02 – 0.05 % (w w^{-1}) [4, 10]. A large part of soil, approximately 95 – 99% is present in the form of insoluble phosphates [11]. Hence, large amount of phosphorus fertilizer are added in the crop soil to increase the phosphorus availability for plant.

Soil supplementation with the form of chemical phosphorus fertilizers containing soluble phosphorus is a way of abundant use. There are many advantages because of a costly and contaminating practice including the highly polluting mode for industrial production requiring the use of sulphuric acid at high temperatures. Excess P application also enhances the potential for P loss to surface waters through overland or subsurface flow causing problems of freshwater eutrophication and erosion [12, 13]. As a result of the chemical reactions from P excess in the soil, these get fixed in the soil. Furthermore, in increased phosphorus concentration from fresh additions in the soil for long period of time, it results in large reserves of fixed P causing a low efficiency of soluble P fertilizers. These reasons are P deficiency in soil. Phosphate solubilizing microorganisms in biofertilizer are offered as a viable alternative to the chemical fertilizers for plant yields because of economical and environmentally safe.

Phosphate solubilizing bacteria (PSB) containing in the phosphorus biofertilizers are an alternative in the prevention of environmental and agricultural issues [14]. They are important solubilizers to assimilate the soil orthophosphatic ion to be soluble phosphorus for plant [15]. The PSB convert the insoluble form of phosphates into soluble forms through the process of acidification, production of gluconic acid, chelation, and exchange reactions [15, 16]. The available phosphates offer soils sustainability for plant health, growth, economic crop yield and soil health.

In ancient times to address such problems and develop sustainable production of rice for local people in area of Suan Pa Tawan agricultural community in Ban Dung, Udonthani province, the use of organic anthill fertilizer to provide essential nutrients for rice growth is common agricultural practice. This fertilizer is a local wisdom in this area for long time. People can make it with themselves by using raw material in their fields. Usage of the organic anthill fertilizer has been improved the fertilizer efficiency by local people from generation to generation in Ban Dung, Udonthani province for long period time. It is worth exploring. These reasons might be possible that the efficient strain of the phosphate solubilizing bacteria (PSB) in the organic anthill fertilizer for their phosphate

solubilizing efficiency may be a part of quest to increase the soil sustainability and ultimately increase in rice yield. Additionally, many studies reported various PSB ecosystem sources such as terrestrial ecosystem especially soil, and water ecosystem including coastal, offshore and mangrove ecosystems [1]. There are not reports to study the present of PSB in the organic anthill fertilizer. Therefore, the objective of this paper focus on isolation of phosphate solubilizing bacteria in the organic anthill fertilizer and investigation of their efficiency to solubilize the different forms of inorganic insoluble phosphates.

2. Materials and methods

Production of organic anthill fertilizer started to collect anthill soils from termite nest from field at area of Suan Pa Tawan agricultural community in Ban Dung, Udon Thani province, Thailand, seeing in Fig. 1. Anthill fertilizer consisted of anthill soil, sterilized rice bran, cooked strictly rice, sterilized soil and water for plant in ratio of 1:1:1:1:2. These ingredients were mixed by hand and mode to be ball shape. The anthill fertilizer balls were fermented at room temperature for 7 days prior to be used as starter for producing liquid anthill fertilizers.

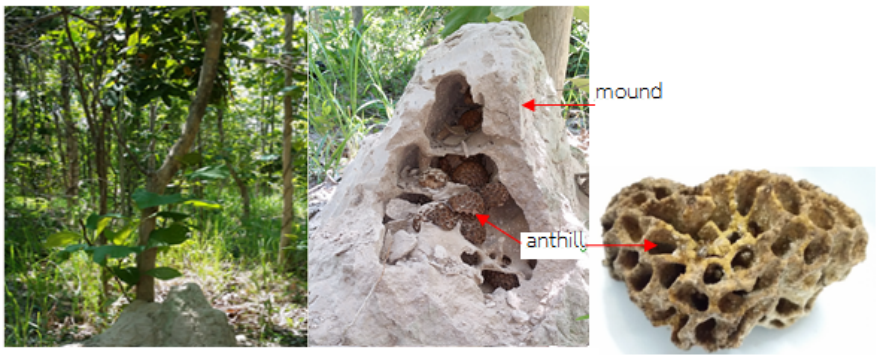


Fig. 1 Anthill position within termite nest and anthill characteristic.

For liquid fertilizer production, the ball starter was added the same mixture to be produced the ball fertilizer but increased water volume. Next, it was fermented at room temperature for 7 days. The liquid fertilizers were determined microbial count, isolation-screening of phosphate solubilizing bacteria (PSB), estimation of PSB colony and cell morphology and finally determination of phosphate solubilizing capability.

Microbial counting indicated microbial population in each liquid anthill fertilizers. It was estimated by plate count method [17]. The liquid fertilizer was diluted with sterile distilled water at a rate of 10^{-4}, 10^{-5} and 10^{-6}. The microbial population in each the diluent was cultured in plate count agar medium by pour plate technique. Culture on plate was incubated at 37 °C overnight. Colony forming appeared and counted them.

PSB isolation started to prepare the serial diluted suspensions at a rate of 10^{-4}, 10^{-5} and 10^{-6} were spreaded on Pikovskaya (PVK) agar medium as selective media for isolating PSB Species [18, 19]. This selective medium contains (per liter) glucose 10 g, $(NH_4)_2SO_4$ 0.50 g, NaCl 0.50 g, KCl 0.20 g, $MgSO_4.7H_2O$ 0.10 g, $MnSO_4$. H_2O 0.001 g, yeast extract 0.5 g, $Ca_3(PO_4)_2$ 5 g and agar 20 g [20]. After 3 – 14 days culture at 37 °C, the colonies appeared on surface of medium. One bacterium having the appearance of a clear phosphate solubilizing zone or clear zone as

phosphate solubilizing property was selected for further purification. Isolates were purified by plating onto the PVK agar medium. Bacterial isolate was freeze-stored in nutrient broth supplemented with 20% (v v^{-1}) glycerol at −20 °C. Further, the isolate was phenotypically characterized for colony and cell morphology.

Bacteria screening. Each isolate screened based on the halo:colony ratio using the spot inoculation method. The diameter of phosphate-solubilizing zone and colony was determined for each colony of isolates. The halo:colony ratio is calculated from the ratio of colony diameter and clear zone diameter of bacteria in PVK agar medium. The clear zone can appear at the surface of the medium. The bacteria with good ability of phosphate solubilization efficiency should express a high halo:colony ratio. This criterion is generally used for preliminary screening of phosphate-solubilizing bacteria including fungi.

Further evaluation for phosphate solubilizing ability of the five isolates was carried out in culture broth medium, PVK broth, with three replications for each isolate. Soluble phosphate and protein in culture broth were determined.

Bacterial morphology. The bacterial isolates form characteristic colonies on PVK agar media. Cell morphology of the isolates was studied by Gram staining [21]. The stained and shape cells were observed under compound microscope. The Gram reaction and cell morphology including characteristic colonies for PSB isolates were recorded.

Phosphate solubilizing capability. The phosphate solubilizing (PS) activity of each of the isolates was assayed by molybdenumblue method [22]. The isolate was cultured in PVK broth containing different insoluble phosphate sources as $AlPO_4$, $Ca_3(PO_4)_2$ and $FePO_4$. Each broth was inoculated with 10% (v v^{-1}) inoculum and cultured at 37 °C for 3 days on an incubator shaker at 200 rpm. The solubilization efficiencies were analysed by method of Olsen and Sommers as well as analysed amount of soluble protein by Lowry method [23]. From each phosphorus source, the same medium without inoculum served as control. From each culture broth, cells and insoluble materials were harvested by centrifugation using a centrifuge at 12,000 rpm for 5 minutes. Soluble phosphate in the supernatant was measured by reaction with ammonium molybdate for phosphorus compounds as ammonium phosphomolybdate and reduced with a compound ascorbic acid to molybdenum blue. Then, the mixture was incubated for 30 minutes at room temperature for color development. Finally, the absorption of light in the wavelength range 595 nm was measured by spectrophotometer.

3. Results and Discussion

Eleven anthill soil samples were analyzed for physical characteristics during collecting anthill soils in forest in area of Suan Pa Tawan agricultural community in Ban Dung, Udonthani province, Thailand. Biological characteristics in form of total bacteria and total phosphate-solubilizing bacteria (PSB) population in the anthill liquid fertilizers were also determined in the Microbiological laboratory at Department of Biological, Faculty of Science, Udonthani Rajabhat University. Data is shown in Table 1. The results showed that the anthill soils were range pH in 5 – 7 and very low in moisture content (0.30 – 1.26%). Number of total bacteria and total PSB in the anthill

liquid fertilizers after 7 days of fermentation were in range of 240 x 10^5 – 950 x 10^5 CFU mL^{-1} and 0.32 x 10^5 – 16 x 10^5 CFU mL^{-1} appearing in plate count agar (PCA) and on Pikovskaya (PVK) agar medium, respectively.

Table 1 Physical anthill soil, total bacteria and PSB population in the anthill liquid fertilizer after 7 days of fermentation

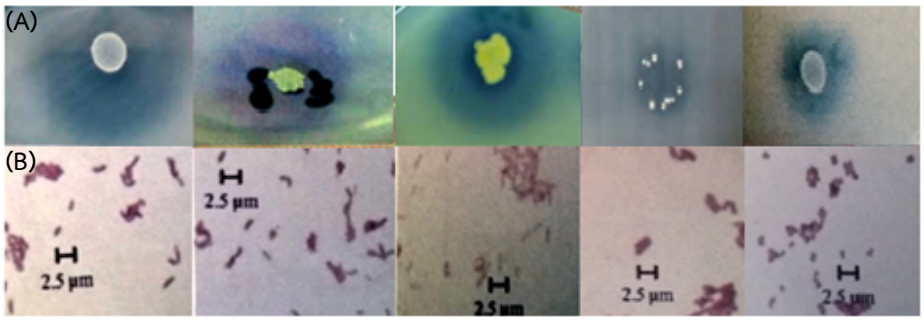
Sampling no.	Physical anthill soil			Total bacteria	Total of PSB
	pH	Temperature (°C)	Moisture content (%)	(CFU mL^{-1}) x 10^5	(CFU mL^{-1}) x 10^5
1	6	29	0.83	550	0.52
2	6	28	1.26	290	8.90
3	6	28	0.33	950	16
4	7	31	0.30	240	0.60
5	5	30	0.66	750	1.56
6	6	29	0.80	500	0.32
7	6	30	0.42	630	4.77
8	6	31	0.17	950	10.90
9	6	30	0.43	550	8.40
10	6	30	0.63	520	3.90
11	6	30	0.74	470	2.13

PSB were isolated from the organic anthill fertilizer samples by using PVK agar medium. A total numbers of 168 PSB isolates were originally obtained and were screened at the first stage of the study. After screening, 25 phosphate-solubilizing bacterial isolates were obtained. These isolates were further screened based on the halo:colony ratio using the spot inoculation technique. The ratio of halo:colony is called in term of hydrolytic capacity (HC). Data is shown in Table 2. The results revealed that the PSB isolates showed range of hydrolytic value from 1 – 4.6. Maximum zone of solubilization considering HC value within 3 days found with isolated PSB code 2-2A (HC4.6), 2-7 (HC3.4), 3-5 (HC3.4), 8-2A (HC3.0) and 8-4 (HC2.9). These five isolates expressed high HC value were further evaluated phosphate solubilizing capability in Pikovskaya broth.

Cell and colony morphology Cells of the five isolates having high HC value were characterized by Gram staining. They also represented distinct types based on differences in colony morphology and colour colony on the PVK agar medium, seeing in Fig. 2. All of isolates were negative strain. Almost isolates were bacillus negative strains, except isolated 8-4 as a coccus negative strain. Their colonies had round- and irregular-shape. The colonies had milk- and yellow-colour. Colony forming was fast developed in the PVK agar medium within 2 days at 37 °C after secondary re-streaking. Similar findings have been observed by Xiufang's study [25], the colonies appeared very well on Aleksandrov medium within 2 days at 30 °C.

Table 2 Phosphate solubilizing bacterial isolates from the organic anthill liquid fertilizers showing colony size, solubilized clear zone and hydrolytic capacity value in PVK agar medium

Isolated PSB code	Diameter of colony	Diameter of clear zone (mm.)	Hydrolytic capacity value
1-1B	0.90	2.50	2.70
1-2A	0.70	1	1.40
2-1	0.80	2.10	2.60
2-2	0.90	1.20	1.30
2-2A	0.60	2.80	4.60
2-7	5	17	3.40
3-1	0.40	1	2.50
3-2	0.80	1.10	1.30
3-4	0.90	1.20	1.30
3-5	6.50	22	3.40
4-3	0.80	1.30	1.60
5-1	0.80	0.90	1.10
6-1	0.90	1.10	1.20
7-1	1.80	2	1.10
8-1	0.80	1	1.20
8-2B	1	1.30	1.30
8-2A	0.10	0.30	3
8-4	3.50	10	2.90
10-2	0.90	1.30	1.40
11-2	2.50	4.50	1.80
15-1	0.60	0.80	1.30
17-1	0.30	0.60	2
19-2	1.90	2	1
19-3A	1.40	1.50	1
19-3B	1.50	1.70	1.10



PSB isolated code 2-2A 2-7 3-5 8-2A 8-4

Fig. 2 (A) Clear zone of phosphate solubilization for the five PBS isolates having the high HC value; (B) Cell shape and Gram's staining

Phosphate solubilizing capability. Each of the five PSB isolates was culture in the PVK broth containing the different insoluble phosphorus form as $AlPO_4$, $Ca_3(PO_4)_2$ and $FePO_4$ for 3 days. After incubation, the amount of released soluble phosphate in the PVK broth by each of PSB isolates was quantitatively determined using chlorostannous reducedmolybdophosphoric acid blue method following method of Jacksonin. Additionally, the amount of released soluble protein was measured by Lowry's method [23] to compare with the soluble phosphate amount. Data in Fig. 3 shows the quantitative estimation of release soluble phosphate concentration contaminating in broth.

The results indicated that the isolates release soluble P from all of inorganic phosphate at the third day of incubation. This result was the same reports of Keneni et al. [24] and Haile et al. [25], solubilization of inorganic non-soluble phosphate was increased with increase in incubation time.

Ironphosphate ($FePO_4$, F-P) solubilization in the studied treatments showed that solubilized range of the F-P was found between 18 to 33 mg L^{-1}. Maximal amount of the soluble phosphate was found in isolate 2-2A (33 mg L^{-1}), 2-7 (30 mg L^{-1}), 3-5 (24 mg L^{-1}), 8-2A (18 mg L^{-1}) and 8-4A (18 mg L^{-1}).

Result of the treatment of aluminiumphosphate ($AlPO_4$, Al-F) solubilization showed that the solubilized range of the $AlPO_4$ was found between 14 to 275 mg L^{-1}. Maximal amount of the soluble phosphate was found in isolate 2-2A (275 mg L^{-1}), 3-5 (56 mg L^{-1}), 8-2A (49 mg L^{-1}), 8-4 (40 mg L^{-1}) and 2-7 (14 mg L^{-1}). For the tricalciumphosphate ($Ca_3(PO_4)_2$) solubilization, the solubilized range of the $Ca_3(PO_4)_2$ was found between 49 to 339 mg L^{-1}. Maximal amount of the soluble phosphate was found in isolate 2-2A (339 mg L^{-1}), 2-7 (180 mg L^{-1}), 3-5 (86 mg L^{-1}), 8-2A (63 mg L^{-1}) and 8-4 (49 mg L^{-1}).

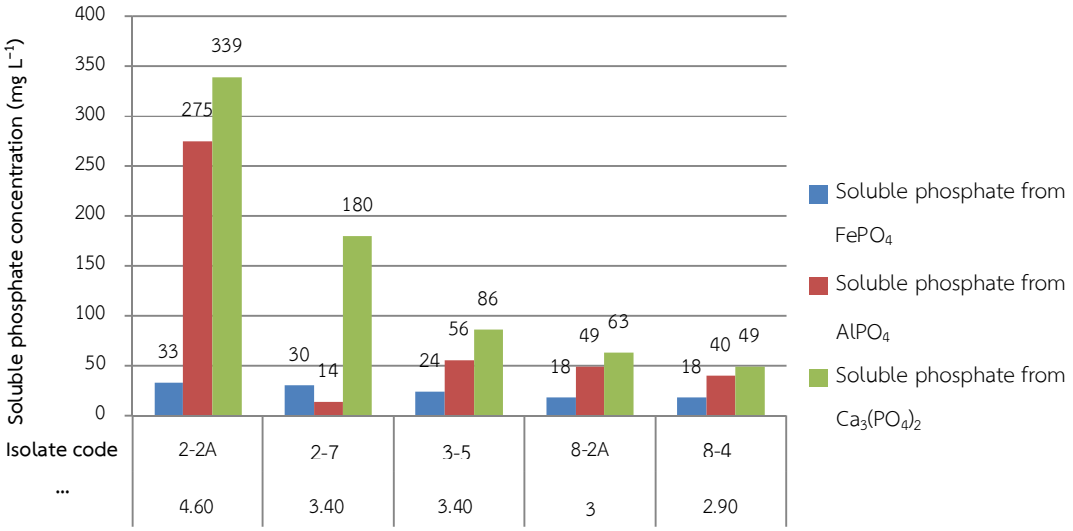


Fig. 3 Released soluble phosphate concentration in culture PVK broth by each PBS isolates having the high HC value

The above results mentioned that the PSB isolates were capable to solubilize Ca-P > Fe-P > Al-P. The 2-2A isolate was found to release P from three inorganic phosphate forms to the tune of 3 – 6 times more than the least P releasing isolates. According with previous studies, they indicated that most PSB strain easily solubilized Ca-P as compared to iron phosphates and rock [26, 27]. For the ability of the isolates to solubilize Fe-P, Asuming-Brempong and Aferi [28] suggested that the PSB produced and released some organic acids, which might have chelating abilities and complexing with iron in the medium. Additionally, the PSB used combinations of mechanisms for solubilisation of the various forms of inorganic phosphates including lowering of pH by acid production, ion chelation, exchange reactions and fermentation in the growth environment.

Considering the relationships between the released soluble protein concentration with soluble phosphate concentration in the PVK broth by measuring in the same broth of all treatments, the result showed that a trend of the protein concentration gradually increased with the increased soluble phosphate during culture in all treatments. Data presents in Fig. 4. According to study of Aseri et al. [29] and Tarafdar et al. [30], they found that phosphate solubilizing microorganisms secreated acid phosphatases and phytases as important enzymes play a role in phosphate solubilization through phosphate assimilation. In the present study, it might be possible that the amount of measured soluble protein was enzymes involving with the process of phosphate solubilization.

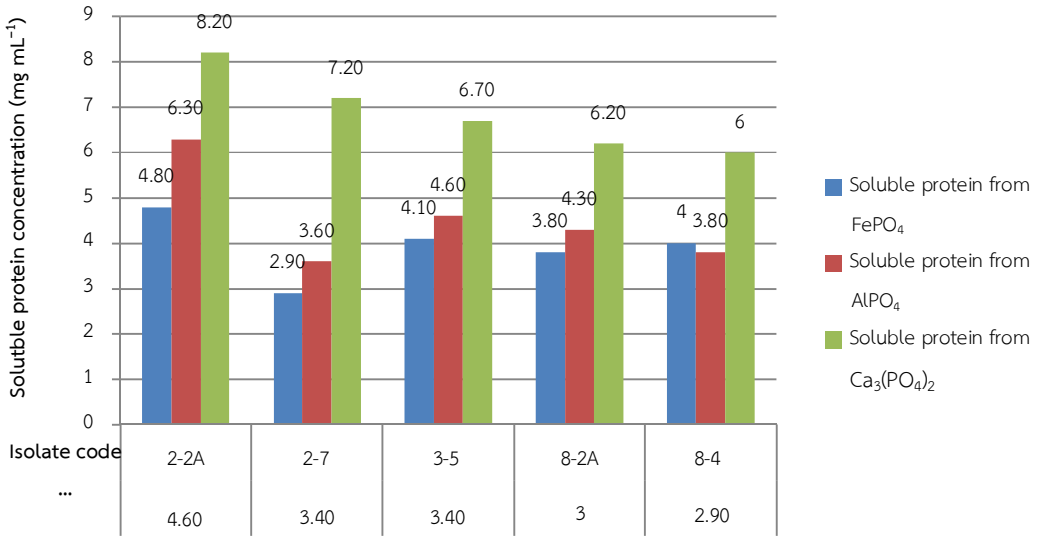


Fig. 4 Released soluble protein concentration in culture PVK broth by each PBS isolates having the high HC value

4. Conclusion

The results from the present study showed that the PSB was a part of microbial ecosystem in the organic anthill fertilizers. Further the present investigation, quantitative phosphate solubilization by different PSB strain being isolated from the organic anthill fertilizer helped to find the efficient strain of PSM. The isolates PSB could solubilize the inorganic non-soluble phosphate forms in the order Ca-P > Al-P > Fe-P. The phosphate solubilizing capacity of these isolates has high efficiency to solubilize phosphate as same as the PSB from other soil source. From the above results, it is concluded that potential of the anthill organic fertilizer as local wisdom people should be supported for the production of eco-friendly phosphate solubilizing biofertilizer for sustainable agriculture.

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