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ISOLATION OF PHOSPHATE SOLUBILIZING BACTERIA FROM ANAEROBIC DIGESTION SLUDGE OF PALM OIL MILL EFFLUENT ON ULTISOLS

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ABSTRACT

Biogas sludge of palm oil mill effluent results from anaerobic digestion from the biogas formation process and contains phosphate solubilizing microorganisms, which can increase phosphorus in the soil. This research aimed to obtain the characteristics and potential of Phosphate Solubilizing Bacteria (PSB) in the biogas sludge from anaerobic digestion of palm oil mill effluent in increasing phosphorus availability in ultisols. These research stages including isolating PSB and obtaining morphological characteristics, ability assessment for solubilizing phosphate, ability to available-P using the completely randomized design within three replications, and determining of organic acids. This research was conducted from January to August 2020. ANOVA and DMRT analyzed data on availability-P at the rate of 5% using SPSS v.21 software. The results showed that the PSB isolates from the biogas sludge dominant had flat elevation, smooth edges, white color, gram-negative, and cell shape of bacillus. The highest index and efficiency of phosphate solubilization were found in the P1 isolate and had the highest available-P from the aluminum phosphate source of 1.42-folds compared to the control. However, the P7 isolate had the highest available-P from the calcium triphosphate and rock phosphate sources (4.62- and 2.66-folds, respectively). It had the highest increase in the available-P in ultisols of 36.21% compared to the control. It also had the highest organic acids in sequence: lactic, oxalic, acetic, and citric acids. These results prove that PSB isolates from biogas sludge can be recommended to increase the availability-P in ultisols.

Keywords: Availability-P; morphological characteristics; phosphate solubilizing bacteria; ultisols.

INTRODUCTION

Phosphorus (P) is a macronutrient that can inhibit the growth and development of plants, especially in tropical areas, due to its low availability in the soil [1]. Phosphorus can contribute 0.2 to 0.8% of dry weight in plants and functions as a constituent of nucleic acids, enzymes, coenzymes, nucleotides, phospholipids, and physiological and biochemical activators such as photosynthesis [2]. The low availability of phosphorus can be caused by one of them is intense fertilization. Continuous fertilization can reduce soil quality and decrease availability-P [3,4]. Therefore, other alternatives are needed to increase the availability-P in the soil, such as phosphate solubilizing microorganisms, especially bacterial groups. It has been reported that the dominant phosphate solubilizing bacteria that can increase the availability-P derived from gram-negative bacteria such as *Pseudomonas* [5,6], *Acinetobacter* [7]. *Pantoea* and *Enterobacter* [8,9], and several gram-positive bacteria such as *Bacillus* [10,11]. Sembiring et al. [12] reported that the use of bacteria of *Burkholderia cepacia* could increase the content of N and K in plants by 8.03% and 10.07%, respectively, compared to the control.

The application of PSB has been widely reported from the various rhizosphere of immature oil palm, mature, and palm oil mill effluent (POME), but there is still lower research on the use of biogas sludge in increasing the availability-P. Food and Agricultural Organization, [13] explained that the biogas sludge is the waste output from the biogas installation from the anaerobic digestion of POME. Adela et al. [14] stated that the effluent or sludge from the installation biodigester is a product of an anaerobic decomposition system that is pathogen-free and has a high nutrient content that can use as organic fertilizer increases soil fertility and the yield of plants. Ohimain et al. [15] reported that the POME is a substrate for biogas production and contains several groups of microorganisms such as *Aspergillus niger*, *Aspergillus flavus, Fusarium, Mucor sp, Penicillin sp, Pseudomonas sp, Serratia sp, Staphylococcus sp,* and *Corynebacterium sp*. Khairuddin et al. [16] stated that the bacteria in the biogas sludge have the potential as a biofertilizer. Mustamu & Priyanto, [17] reported that the biogas sludge contained the PSB with a population of 42×10^4 CFU/ml. It had the potential as biological fertilizer to provide phosphate in the soil.

Limited information is available regarding the potential of biofertilizers derived from biogas sludge. It is necessary for basic research to determine the characteristics and potential of PSB in the biogas sludge from anaerobic digestion of palm oil mill effluents in increasing the availability-P in ultisols.

MATERIALS AND METHODS

Research Sites

Location researched at the Laboratory of Soil Biology, Faculty of Agriculture, Universitas Sumatera Utara, Medan. Solubilizing phosphate was tested at the Service Laboratory of Indonesian Research Institute for Biotechnology and Bioindustry, Serpong, South Tangerang City, Banten. Physicochemical characteristics of ultisols were analyzed at the Analytical Laboratory of PT. Socfin Indonesia, Medan. Determination of organic acids was analyzed at the Laboratory of Agrochemical Residues, Bogor. This research was conducted from January until August 2020.

Isolates Source

The biogas sludge sample used as a phosphate solubilizing isolate was obtained from the anaerobic reactor of POME in the PT. Nubika Jaya, Blok Songo, Pinang City, Labuhanbatu District, North Sumatra, Indonesia (100°06'16.61"E, 01°56'45.32"N).

Medium Preparation of Pikovskaya

Prepared and weighed the ingredients such as $Ca_3(PO_4)_2$ 5 g; $(NH_4)_2SO_4$ 0.5 g; NaCl 0.2 g; $MgSO_4.7H_2O$ 0.1 g; KCl 0.2 g; glucose 10 g; yeast extract 0.5 g; agar 20 g; MnSO4 and $FeSO_4$ 0.1 g, 1000 ml distilled water [18] with analytical scales and dissolved with distilled water, then homogeneous the medium solution using a hotplate.

Isolation of Phosphate Solubilizing Bacteria (PSB)

It suspended 1 ml of bacteria from the isolate obtained and inserted into a test tube containing 9 ml of distilled water and homogenized. Serially, 1 ml of the suspension from the previous dilution was added to 9 ml of distilled water. The dilution was made up to 10^{-5}. Resuspended 0.1 ml of the final dilution is spread over pikovskaya medium aseptically. The culture medium was incubated for 2 to 3 days at room temperature. The growth of phosphate solubilizing microbes is characterized by a halozone around the colonies on the pikovskaya medium. Bacteria that grow on the pikovskaya medium are selected to be purified and tested.

Morphological Characteristics of PSB Isolates

One ml of each dilution was taken using a micropipette then inoculated on a petridish containing the pikovskaya medium by the pouring method. The biogas sludge suspension was incubated at 35^0C for 48 h until a growing colony does achieve. The selection of purified microbial colonies does based on the differences in the appearance of colony morphology, including shape, elevation, edges, color, gram staining, and cell shape, to obtain pure isolates (Fig. 1). Determination of gram staining and bacterial cell shape was conducted by staining crystal violet, iodine, 95% alcohol, and safranin and then observed using a stereo microscope with the magnification of 400x. Gram-positive bacteria are shown in purple cells, and gram-negative bacteria are shown in red cells.

The P-Solubilization Ability of PSB

Seven bacterial isolates were obtained and purified and then tested to dissolve phosphate to determine their solubility index. A total of 1 inoculating loop was spotted on pikovskaya medium aseptically and incubated for seven days at room temperature. The halozone diameter formed is measured and calculated to determine the Phosphate Solubilization Index (PSI) and Phosphate Solubilization Efficiency (PSE) through equations 1 and 2. The PSI and PSE values are obtained from the comparison between the halozone diameter formed with the colony diameter [19], and the ability of PSB isolates was tested to dissolve P-insoluble from the three phosphate types ($Ca_3(PO_4)_2$, $AlPO_4$, rock phosphate).

$$PSI = \frac{\text{Colony diameter} + \text{Halozone diameter}}{\text{Colony diameter}} \quad (1)$$

$$PSE = \frac{\text{Halozone diameter}}{\text{Colony diameter}} \times 100\% \quad (2)$$

The classification of phosphate solubilization index was determined based on Filho & Vidor, [20] with the PSI value of <1 was classified as very low; the PSI value ranged from 1 until 2 was classified as low; the PSI value ranged from 2 to 3 was classified as moderate, the PSI value of ≥ 3 was classified as high.

Potential Test of PSB Isolates for Phosphorus Availability in Ultisols

The soil sample was taken from the Simalingkar area, Medan Tuntungan Subdistrict, Medan City with soil type of ultisols, and analyzed several physicochemical properties. The analysis results showed that the dominant ultisols properties were very acidic for soil pH, very low until low for exchangeable-Ca, exchangeable-Na, exchangeable-Al, organic-C, total-N, exchangeable-K, and exchangeable-Mg (Table 1). The isolates were purified and cultured in medium Nutrient Broth (NB) for two days. Ultisols was sterilized using autoclave and weighed 100 g, then put into an Erlenmeyer flask, and one ml of the isolate was inoculated and incubated for 14 days. The culture was shaken at 100 rpm periodically. This experiment was conducted using a completely randomized design within three replications. At the end of incubation, the available-P was determined by the spectrophotometer method and compared to the control.

Determination of Organic Acids

Organic acid determination was identified only for the P7 isolate, which had the highest available-P compared to other isolates. The extraction of organic acids from PSB was obtained in the following: sample weighed 0.5 to 1 ml from one test tube, added 10 to 20 ml 0.1 N NaOH for 12 h, filtered through Whatman No.42 filter paper and centrifuged at 3,000 to 5,000 rpm for 15 min, the supernatant was acidified to pH 2.5 with 1 N HCl to precipitate the humic acid, then withdraw for 16 h, the mixture is centrifuged at 3,000 until 5,000 rpm for 15 min, the organic acids are obtained by extracting the supernatant three times with 10 ml of ethyl acetate for 5 min, evaporating the solvent to dry in a rotary evaporator at 40□ and redissolve the residue using 0.01 NH_2SO_4 solvent and injected with the HPLC [21].

Table 1. Physicochemical properties of ultisols before application of PSB isolates

Ultisols physicochemical	Value	Category
Soil texture		Clay loam
% sand	43.0	
% silt	28.5	
% clay	28.5	
Soil pH		
Actual (H_2O)	4.10	Very acidic
Potential (KCl)	3.46	
Organic-C (%)	1.97	Low
Total-N (%)	0.17	Low
Available-P (mg kg^{-1})	135.90	Very high
Cation exchangeable capacity (meq/100 g)	31.06	High
Exchangeable cations		
K (meq/100 g)	0.35	Low
Ca (meq/100 g)	0.46	Very Low
Mg (meq/100 g)	0.63	Low
Na (meq/100 g)	0.06	Very Low
Al (meq/100 g)	0.05	Very Low

Data Analysis

The ability of PSB isolates to increase available-P was analyzed by ANOVA at the level of 5% using the SPSS v.21 software. If the treatment is influential, proceed to DMRT at 5% [22].

RESULTS AND DISCUSSION

Morphological Characteristics of PSB Isolates

Based on the isolation and selection of PSB, seven pure isolates from biogas sludge (Fig. 1) with morphological characteristics could be seen in Table 2. The shape of PSB isolates from biogas sludge varies widely (circular, irregular, and filamentous). The dominant isolates (P1, P3, P4, P7) had flat elevations, and other isolates (P2, P5, P6) had convex elevations. The dominant types of isolates (P2, P5, P6) had smooth edges, two isolates (P1, P3) had irregular edges, and one isolate with lobate edges (P4). There were six types of isolates (P1-P4, P6-P7) that had a similar color (white) and one isolate (P5) was a yellow color. It was seen that the dominant isolates were classified as gram-negative bacteria (P1, P4, P5, P6) and three types of isolates (P2, P3 P7) were classified as gram-positive bacteria. The dominant PSB isolates obtained the cell shape of a bacillus (P2, P3, P5-P7) and one isolate had the cell shape of streptobacillus (P1) and one isolate was coccus (P4).

The PSB isolates from the biogas sludge dominant had flat elevation, smooth edges, white color, gram-negative, and cell shape of bacillus. It was indicated that there are isolates that have a cell wall structure with thick peptidoglycan content (gram-positive) and high lipid content (gram-negative), and Kumar et al. [23] reported that gram-negative bacteria more effectively solubilizing phosphate compared to the gram-positive due to more diverse to release organic acids in the soil. All the PSB isolates from the biogas sludge can solubilize phosphate, which was characterized by the halozone that was higher than

Table 2. Morphological characteristics of PSB isolates from the biogas sludge

Isolates	Colony Shape	Colony Elevation	Colony Edge	Colony Color	Gram staining	Cell Shape
P1	Irregular	Flat	Irregular	White	-	Streptobacillus
P2	Circular	Convex	Smooth	White	+	Bacillus
P3	Irregular	Flat	Irregular	White	+	Bacillus
P4	Irregular	Flat	Lobate	White	-	Coccus
P5	Circular	Convex	Smooth	Yellow	-	Bacillus
P6	Circular	Convex	Smooth	White	-	Bacillus
P7	Filamentous	Flat	Filamentous	White	+	Bacillus

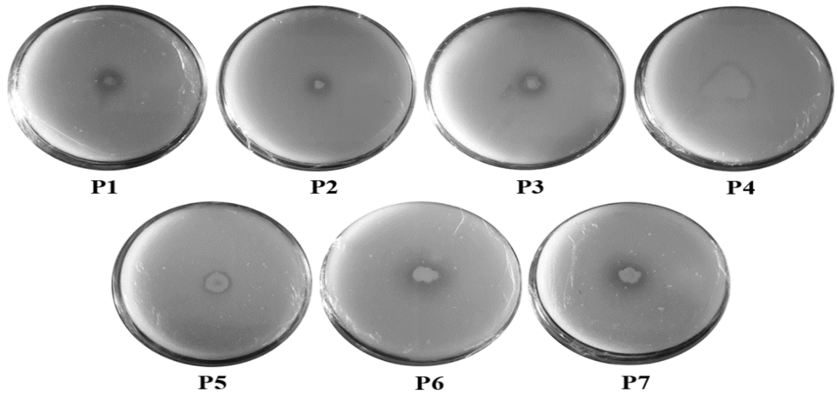

Fig. 1. Pure isolates of PSB from the biogas sludge

the colony diameter (Table 3). Baharuddin et al. [24] reported that bacteria of *Geobacillus pallidus* (thermophilic) was found in Empty Fruit Bunch (EFB) and POME compost with morphological characteristics of bacillus shape, flat elevation, cream color, gram-negative, and length from 1 to 14 µm. Dermiyati et al. [25] reported 67 aerobic isolates, 38 facultative anaerobic isolates, 61 anaerobic isolates from 166 isolates of PSB in the Fresh Fruit Bunches (FFB) of oil palm. Zainudin et al. [26] reported that PSB obtained from EFB + sludge such as strains of *Corynascus sp, Scytalidium sp, Chaetomium sp, Scopulariopsis sp, Bacillus sp,* and *Streptomyces sp.* Wan et al, [27] reported that 18 strains of PSB were obtained, which had the ability to P-solubilize sources, and all of these strains were included in eight genera with the gram-negative bacteria more dominant (genera *Massilia, Cupriavidus, Stenotrophomonas, Acinetobacter, Pseudomonas, Ochrobactrum*). Meanwhile, the gram-positive bacteria were genera *Bacillus* and *Arthrobacter*. George et al. [28] also stated that PSB would dissolve phosphate in the form of PO_4 using the phosphatase enzyme, forming a halozone diameter around the bacterial colonies.

The P-Solubilization Ability of PSB

Colony diameter, halozone, index, and efficiency of phosphate solubilization (PSI and PSE) in the seven isolates of PSB from biogas sludge could be presented in Table 3.

Table 3 showed that the addition of the halozone diameter to all isolates of PSB from biogas sludge. It will affect the index and efficiency of phosphate solubilization in the seven isolates from the biogas sludge with the highest PSI and PSE was found in the P1 isolate (moderate) compared to other isolates (P2-P7) had the PSI were classified as low. It was indicated that all the PSB isolates from

Table 3. P-solubilization ability from seven isolates of PSB from the biogas sludge

Isolates	Colony diameter (mm)	Halozone diameter (mm)	PSE (%)	PSI	Classification PSI
Control	-	-	-	-	-
P1	0.55	1.25	227.27	2.27	Moderate
P2	0.60	1.10	183.33	1.83	Low
P3	0.90	1.40	155.56	1.55	Low
P4	2.20	2.30	104.55	1.04	Low
P5	1.20	1.35	112.50	1.12	Low
P6	1.30	1.75	134.62	1.34	Low
P7	1.00	1.40	140.00	1.40	Low

Note: the PSI value of <1 was classified as very low; the PSI value ranged from 1 until 2 was classified as low; the PSI value ranged from 2 until 3 was classified as moderate, the PSI value of ≥ 3 was classified as high. (Filho & Vidor, 2000) the biogas sludge could convert the phosphate from insoluble into soluble compounds. It could be seen from the ability of all isolates to increase the potential for solubilizing P-insoluble (Table 4). These results are similar with Pande et al. [29] reported that colonies that show halozone diameter around bacterial growth can dissolve phosphate and found in three from eight isolates that had PSI of ≥ 4, including isolates of C1 (4.88); H6 (4.64), and A4 (4.48). Paul & Sinha, [30] reported that the formation of halozone diameter around bacterial colonies could be caused by the production of organic acids, polysaccharides, and the activity of the phosphatase enzyme from the strains of PSB. Irawan et al. [31] reported that the bacterial strains isolated from the oil palm rhizosphere could solubilize $Ca_3(PO_4)_2$ by increasing the halozone diameter. Dermiyati et al. [25] reported that bacterial isolates in the FFB of oil palm that could phosphate solubilize were classified as high to very high (nine aerobic isolates, five facultative anaerobic isolates, three anaerobic isolates). Khan et al. [32] stated that PSB could convert the insoluble phosphorus into soluble compounds (HPO_4^{2-} and $H_2PO_4^-$) through organic acid production, chelating reactions, and ion exchange.

In contrast, the PSB isolates of P2 and P3 (gram-positive) had higher PSI and PSE than gram-negative isolates. It was caused by the pikovskaya medium used affects the halozone for P2 and P3 isolates. It was confirmed by Chen et al. [33] stated that the ability of bacteria to soluble phosphate depends on the substrate, medium, temperature, time, and mechanism of organic acid production. Yu et al. [34] also reported that the fluctuation in pH by some strains may be due to the chelation of organic acids with calcium ions (Ca^{2+}) in the $Ca_3(PO_4)_2$ and it had a strong negative correlation between soluble phosphorus content and pH in the culture medium.

It could present the P-solubilization ability of PSB from biogas sludge qualitatively in Table 4.

Table 4 showed that the P1-P7 isolates could solubilize phosphorus from the three sources of P-insoluble (calcium triphosphate, aluminum phosphate, and rock phosphate) compared to the control. The P7 isolate had the highest ability to solubilize phosphorus from calcium triphosphate and rock phosphate sources by 4.62 and 2.66 folds, respectively, compared to the control. The P1 isolate had the highest ability to solubilize phosphorus from an aluminum phosphate source of 1.42-folds compared to the control. The high solubility of phosphate was caused by a pH decrease in all PSB isolates (P1 to P7) from the biogas sludge compared to the control. The decrease in pH causes the high organic acids produced (Table 5). The results are similar to Selvi et al. [35] the primary mechanism of phosphorus solubilizing was a decrease in soil pH with the production of organic acids or the protons release, resulting in the insoluble phosphate (calcium phosphate, rock phosphate, aluminum phosphate) will increase in solubility with a decrease in soil pH. Khan et al. [36] reported that the organic acids produced by PSB chelate the cations absorbed in the phosphate to convert them into soluble phosphate compounds. Kumar et al. [23]; Satyaprakash et al. [37] Yousefi et al. [38]; Ahmed & Shahab, [39] reported that organic acids that can solubilize phosphate include citric, lactic, gluconic, 2-ketogluconic, oxalate, glyconic,

Table 4. The P-solubilization ability of PSB from the three sources of insoluble phosphorus by seven isolates from biogas sludge

Isolates	Calcium Triphosphate (ppm)	pH	Aluminum Phosphate (ppm)	pH	Rock Phosphate	pH
Control	206	6.1	223	6.0	186	6.6
P1	410	4.4	318	3.6	230	4.9
P2	895	4.6	306	4.1	291	5.1
P3	432	4.7	285	3.7	279	5.0
P4	766	4.7	306	4.1	306	5.7
P5	807	5.5	256	5.3	266	5.7
P6	914	4.6	268	4.5	196	4.8
P7	952	4.5	293	4.1	494	5.2

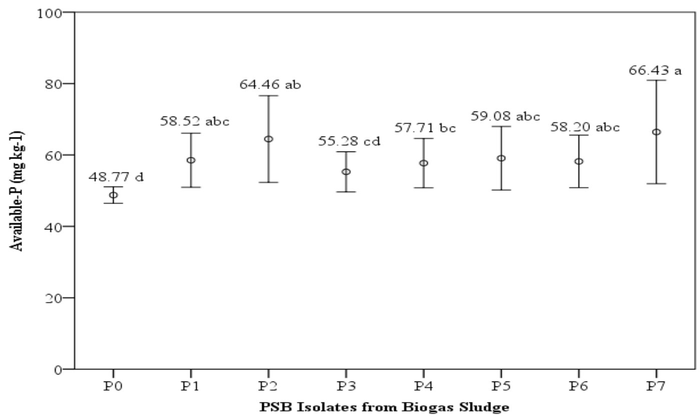

Fig. 2. Effect of PSB isolates from the biogas sludge on the available-Pof ultisols. Means followed by the different letters are significantly different with the DMRT at 5% ± standard error.

acetate, malate, fumarate, succinic, tartaric, malonic, glutaric, propionate, butyrate, glyoxalate, and adipic acids. Walpola & Yoon, [40] reported that the organic acid of 2-ketogluconic was strongly calcium chelator. Babalola & Glick, [41] noted that the bacteria *Pseudomonas spp, Agrobacterium spp,* and *Bacillus circulans* could be mobilized of insoluble phosphorus by solubilizing and mineralization. Suleman et al. [42] also reported that two of 15 PSB strains, namely *Pseudomonas* sp. MS16 and *Enterobacter* sp. MS32 efficiently solubilizes phosphorus with the PSI ranged of 3.2 until 5.8.

The Ability of PSB on the Available-P in Ultisols

PSB isolates from the biogas sludge significantly increased the available-P of ultisols (Fig. 2).

Fig. 2 explained that the P7 isolate could increase the highest available-P content in ultisol by 66.43 mg kg^{-1} compared to other isolates and the effect was 36.21% compared to the control. It was influenced by the decrease in pH in solubilizing phosphate compared to the control (Table 4) and the organic acids produced by isolate P7 (Table 5). According to Richardson & Simpson, [43]; Khan et al. [44] stated that PSB produced organic acids that solubilize unavailable phosphorus (PO_4^{3-}) into available-P (HPO_4^{2-}, $H_2PO_4^-$) and Perez et al. [45] reported a decrease in pH from 7 to 2 during the acidification process. Egamberdiyeva, [46] reported that the PSB strains of *Pseudomonas alcaligenes* PsA15, *Bacillus polymyxa* BcP26, and *Mycobacterium phlei* MbP18 had the greater stimulating effect on the absorption of nitrogen (N), phosphorus (P), and potassium (K) in nutrient deficient in soil due to phosphate solubility and organic acid production. Shi et al. [47] reported that the PSB could be increased available-P by 3.90-folds compared to the control. Walpola & Yoon, [48] also reported that the PSB-1 strain could be increased available-P by 58.83% compared to the control.

Organic Acids Concentration

The analysis results of organic acids produced by isolate P7 could be presented in Table 5. The results showed that the P7 isolate had organic acids such as acetic, lactic, citric, and oxalic acids, but was not found for malic acid.

Table 5. Organic acids produced by P7 isolate from the biogas sludge

No	Organic acids	Concentration (mg kg^{-1})
1	Acetic Acid	1.396
2	Lactic Acid	3.453
3	Citric Acid	0.555
4	Malic Acid	nd
5	Oxalic Acid	2.773

Note: nd = not detected

Table 5 explained that the P7 isolate had the highest organic acids sequentially include lactic, oxalic, acetic, and citric acids. The production of organic acids in the P7 isolate from the biogas sludge was influenced by a decrease in pH at that solubilizing insoluble-P (Table 4) and the resulting halozone diameter (Table 3). The result was similar with Tahir et al. [49] reported that the PSB strain *Bacillus* T-34 using all carbon sources (glucose, galactose, maltose, and sucrose) to produce citric, malic, acetic, and lactic acids with higher concentrations compared to the strains *Azospirillum* WS-1 and *Enterobacter* T-41. Wei et al. [50] also reported that the inoculation of PSB could be affected pH, total acidity, and production of oxalic, lactic, citric, succinic, acetic, and formic acids.

CONCLUSION

The phosphate solubilizing bacteria isolated from the biogas sludge dominant had flat elevation, smooth edges, white color, gram-negative, and cell shape of bacillus. The highest index and efficiency of phosphate solubilizing was found in P1 isolates (moderate) compared to other isolates (P2-P7) had the PSI were classified as low. The P7 isolate had the highest ability to solubilize phosphorus from calcium triphosphate and rock phosphate (4.62 and 2.66-folds), whereas the P1 isolate had the highest ability to solubilize phosphorus from aluminum phosphate source by 1.42-folds compared to the control. The P7 isolate could increase the available-P content of ultisol by 36.21% compared to the control, and it had the highest organic acids sequentially, such as lactic, oxalic, acetic, and citric acids.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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