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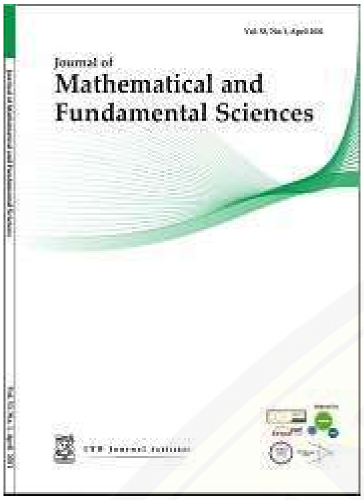

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Phosphate Solubilizing Bacteria Adaptive to Vinasse

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Abstract. Microorganisms identified as phosphate solubilizing bacteria (PSB) adaptive to vinasse were successfully screened from sugarcane soil from an agriculatural estate in Jatiroto. By conducting a screening on Pikovskaya's agar medium (PAM), we found that five different isolates were detected as PSB (pvk-5a, pvk-5b, pvk-6b, pvk-7a, and pvk-8a). Of the five isolates only three could be grown and were found to be adaptive to vinasse based medium without any nutrients added (pvk-5a, pvk-5b and pvk-7a). The three isolates were characterized as coccus and Gram negative with no endospores detected. We suggest that these three isolates can be used as biofertilizer agent to support organic farming.

Keywords: *bacillus; biofertilizer; Gram negative; PSB; vinasse.*

1 Introduction

Soil microorganisms known as phosphate solubilizing bacteria (PSB) are found in nature within the plant rhizosphere area. This type of microorganism has the ability to solubilize phosphate sediment [1]. In field application, PSB are often used as biofertilizer agent to increase the efficiency of phosphate fertilizers [2-4]. The availability of PSB in soil may elevate the phosphate uptake by the plant and improve the yield of the crop [5]. As for cultivation, PSB can be grown on medium containing $Ca_3(PO_4)_2$, $FePO_4$, $AlPO_4$, rock phosphate and any other inorganic phosphate mineral [6]. However, to produce and grow large quantities of PSB as biofertilizer using this medium, the potential high cost due to the material used in the production process of PSB must be considered. To overcome this problem, material substitution which may reduce the production cost of PSB must be investigated. Gómez and Rodríguez found in a previous study that a huge amount of residual organic-rich vinasse was released during ethanol production from molasses [7]. From one liter of ethanol produced, around 13-30 l of vinasse was released as waste. Also, Jiang *et al.* has reported that direct application of vinasse to agricultural land can increase the productivity of plants [3]. Based on the economic potential of vinasse corroborated by these studies, we decided to further investigate vinasse as a PSB production medium. Addition of PSB to vinasse was considered to be a logical approach in this research to extend the quality of vinasse as organic

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fertilizer. Therefore we focused this study on screening PSB microorganisms that are adaptive to vinasse. Furthermore, this research was expected to justify that, in turn, the vinasse can be utilized as a cheap raw material for PSB production.

2 Material and Method

Soil samples as PSB source were randomly collected in 5 locations within 7.2 h from sugarcane soil from an agricultural estate in Jatiroto, East Java, which was treated with vinasse as fertilizer. All sampling was done in duplicate. Twenty five grams of soil sample at every location was directly suspended in 225 ml of sterilized water containing 0.85% NaCl and stored at 4°C as PSB screening source.

The suspended samples were then diluted from 10-1 to 10-8 [8], plated to Pikovskaya's agar medium (PAM) and incubated at 30°C for isolation and the screening process. The presence of PSB in the PAM medium is indicated by a clear zone around colonies, grown after 48 hours of incubation. To obtain a single species, inoculation was repeated by picking up one loop of a single colony, which was directly plated in the same medium. The single colony of PSB isolates was stored at 4°C for further analysis.

The index activity of all PSB isolates was assayed using the same medium, grown and observed after incubation for 72 h at 30°C. The index activity of the PSB was then calculated by comparing the ratio between clear-zone and colony diameter [9]. All isolates of high index activity were selected for further analysis.

The selected PSB isolates were also characterized and evaluated based on colony forming units on the PAM. Macroscopical observation involved colony shape, surface, elevation, edge, concentric and radial line, and growth of colonies. Microscopical observation was based on Gram staining and endospore detection [10].

Selection of PSB adaptive to vinasse was carried out by culturing of all potent isolates on diluted vinasse medium without any nutrients added. Distillated water was used for dilution. Dilution factors (v/v = vinasse/water) used were 1:2 and 1:3). All cultures were observed and the total colony number was counted, expressed as colony form units (CFU). As control, Pikovskaya's liquid medium (PLM) was used. The selection was completed in 72 hours, after which the total colony was periodically monitored (every 4 hours).

3 Results and Discussion

Nine species of bacteria were isolated from sugarcane soil from an agricultural estate in Jatiroto. Among them only five isolates were identified as PSB (pvk-5a, pvk-5b, pvk-6b, pvk-7a and pvk-8a) as shown at Figure 1 below.

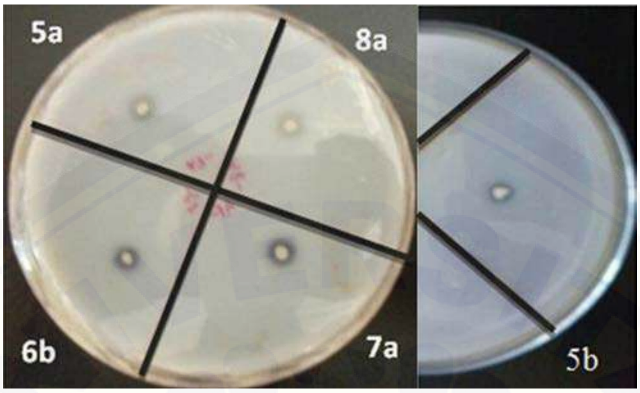

Figure 1 Five different isolates (pvk-5a, pvk-5b, pvk-6b, pvk-7a and pvk-8a) that produced a clear zone around the CFU in PAM after 72 hours incubation at 30°C were detected as PSB.

The presence of a clear zone around the colony indicated that five isolates secreted an extracellular enzyme hydrolyzed organic acid and released Ca^{++} from $Ca_3(PO_4)_2$ through which their hydroxyl and carboxyl groups chelate the cations bound to phosphate, thereby converting it into soluble form. In addition, H_2PO_4 is released to the clear-zone area from other insoluble organic or inorganic phosphate substances. The organic acid released involves citric acid, glutamic, succinate, lactate, oxalate, glyco-oxalate, malate, phumarate, tartarate or alpha-cetoglutaric acid [11,4]. It has also been reported that the principal mechanism of mineral phosphate solubilization is hydrolysis process of organic phosphorus in soil by some organic acids and acid phosphatases, secreted by PSB [6,12].

Table 1 Summary of activity index of PSB isolates after 72 hours of incubation.

Isolate	Activity Index
pvk-5a	2,01
pvk-5b	1,52
pvk-6b	1,01
pvk-7a	1,65
pvk-8a	1,58

As shown in Table 1, the ability of each isolate to secrete inorganic extracelllular acid resulted in different activity indexes. The isolate pvk-5a produced the highest activity index (2.01) followed by pvk-7a, pvk-8a, pvk-5b and pvk-6b respectively after 72 hours of incubation at 32°C.

Macroscopical and microscopical observation revealed that all PSB isolates showed different colony shapes when grown in PAM. However, there were only few differences concerning the elevation and edge of the colonies. All isolates consisted of similar yellowish white colored colonies with the entire colony being opaque with a concentric line while grown on the surface of the medium. Microscopical observation of all isolates showed coccus type bacteria, no Gram staining, no endospores and non motil, as described in Table 2. Based on these observations, all isolates can be classified into the same genus as *Bacillus sp*.

Table 2 Macroscopical and microscopical observation of PSB.

No	Characteristic of PSB isolate	Isolate				
		pvk-5a	pvk-5b	pvk-6b	pvk-7a	pvk-8a
1	Colony shape					
	Plate culture	Irregular	Curled	Irregular	Irregular	Curled
	Slant culture	Echinulated	Echinulated	Echinulated	Echinulated	Echinulated
	Straight culture	Beaded	Beaded	Echinulated	Beaded	Beaded
2	Surface	Unshine	Unshine	Unshine	Unshine	Unshine
3	Elevation	RCBE	RCBE	RCBE	Umbonated	Umbonated
4	Edge colony	Undulate	Crenate	Crenate	Undulate	Undulate
5	Entire	Opaque	Opaque	Opaque	Opaque	Opaque
6	Colony color	Yellowish white	White milk	Yellowish white	White milk	Yellowish white
7	Growth	Surface	Surface	Surface	Surface	Surface
8	Colony liner	Concentric	Concentric	Concentric	Concentric	Concentric
9	Cell shape	Coccus	Coccus	Coccus	Coccus	Coccus
10	Gram character	Negative	Negative	Negative	Negative	Negative
11	Endospores	-	-	-	-	-

The adaptation ability of PSB isolates that grow in vinasse based medium without any nutrients added was observed through proliferation, as shown in Figure 2. For this purpose, PLM was used as control. The investigation resulted in only three isolates that were able to grow on vinasse containing medium (pvk-5a, pvk-5b and pvk-7a). These three isolates seemed to be adaptive PSB utilizing carbon and nitrogen sources as well as macro and micro nutrients from vinasse for their growth, although they gave optimum population growth at different incubation times. Optimum growth for pvk-5b and pvk-7a was at 12-16 hours of incubation, whereas pvk-5a needed a longer incubation time (24 hours) if vinasse with a dilution factor of 1:2 was used. However, all isolates could not grow if vinasse with a dilution factor of 1:3 was used.

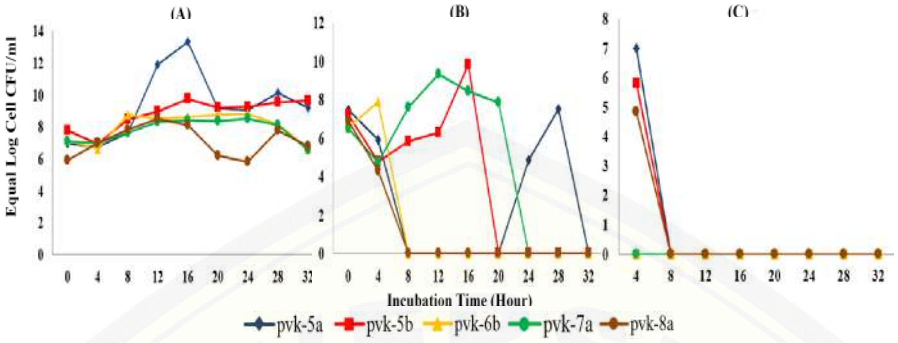

Figure 2 Growth pattern of PSB after 32 hours incubation in Pikovskaya's liquid medium (A), vinasse medium diluted 1:2 (B) and vinasse medium diluted 1:3 (C), and without any minerals or nutrients added.

Some researches have been done related to isolation and characterization of PSB [4,6], including their efficiency to produce and release phosphate in soil [12]. Researchers expect to get beneficial PSB microorganisms for the sustainable use of phosphates in agriculture [12,13]. However, techniques to increase or multiply PSB in soil have received less attention. As described in this research, vinasse has been elucidated as a resource for PSB cultivation medium. Evalution of PSB growth in vinasse against pH, temperature, and other parameters as well should still be further evaluated. Then, a PSB-rich vinasse product is expected that can be utilized on agricultural land as a cheap biofertilizer.

4 Conclusion

Five bacterial isolates of soil treated with vinasse from an agricultural estate were identified as PSB. Among them only three isolates were able to adapt and grow in vinasse liquid medium with a 1:2 dilution factor (pvk-5a, pvk-5b and pvk-7a). The three isolates were characterized as coccoid bacteria, Gram negative with no endospores detected. According to the results, there is strong evidence that the three adaptive PSB isolates had the ability to solubilize both mineral and organic phosphates. The vinasse itself is suited to be used as cheap medium source for PSB production, which may be utilised to support organic farming. This may elevate the phosphate uptake of plants and therefore increase the crop yield significantly.

Acknowledgements

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