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Isolation and identification of phosphate solubilizing bacteria from indigenous microorganisms (IMO) of cow rumen in East Java, Indonesia as eco-friendly biofertilizer

Meli Astriani[1,2], Siti Zubaidah[1]*, Abdul Latief Abadi[3] and Endang Suarsini[1]

[1]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Malang, Jalan Semarang, Malang 65145 Jawa Timur, Indonesia.
[2]Department of Biology Education, Faculty of Teacher Training and Education, Universitas Muhammadiyah Palembang. Jl. Jenderal Ahmad Yani 13 Ulu, Palembang 30116, South Sumatera, Indonesia.
[3]Departement of Plant Pests and Diseases, Faculty of Agriculture, Brawijaya University, Jalan Veteran, Ketawanggede, Malang 65145 Jawa Timur, Indonesia.
Email: siti.zubaidah.fmipa@um.ac.id

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ABSTRACT

Aims: Phosphate is an essential nutrient required for plant growth, but its solubility in the soil is relatively low (0.1%). Microbes can dissolve phosphate to meet crop requirements. This study aimed to isolate phosphate solubilizing bacteria from indigenous microorganisms (IMO) of cow rumen.
Methodology and results: The selection of isolates on a Pikovskaya medium was using a clear zone index and a spectrophotometer for phosphate solubilization measurements. Hypersensitivity was tested on tobacco leaves and tested antagonists within isolates. The results found that four selected isolates had the highest phosphate dissolving potential, namely, MTA1, SMAD1, SMAD2, and SMAD3. The culture of selected isolates on plate media showed that the morphological characters of the four colonies are the same. They had round form (circular), the edge of the colony were smooth, flat elevation, white and cream color. Isolate MTA1 had the highest phosphate solubilizing activity compared to the others. The isolate that showed the highest phosphate solubilizing activity were identified based on 16S rRNA gene. The result of molecular identification showed that strain MTA1 was closely related to *Lactobacillus plantarum* with a similarity level of 99%. *L. plantarum* performed the highest ability to form a clear zone (7.66 mm). The highest concentration of soluble phosphate was observed on day 5 (278.42 mg/L).
Conclusion, significance, and impact of the study: *Lactobacillus plantarum* which was isolated from the IMO of cow rumen in East Java, Indonesia was identified as one of the phosphate solubilizing bacteria that are useful for the development of eco-friendly biofertilizer. The application of phosphate solubilizing microbes can be used to increase the soil fertility.

Keywords: Phosphate solubilizing bacteria, indigenous microorganism, *Lactobacillus plantarum*, eco-friendly biofertilizer

INTRODUCTION

Phosphate (P) is a macronutrient essential for plant growth. It plays a crucial role in plant metabolic processes, including energy transfer, signal transduction, respiration, macromolecular biosynthesis, and photosynthesis (Anand *et al.*, 2016). However, phosphate solubility in the soil is relatively low, only about 0.1% of the total P available for plants (Sharma *et al.*, 2013). Phosphate, which can easily react with iron, aluminum, and calcium, produces phosphate deposition in the soil so that it is rarely found as a free element. Therefore, to make phosphate available to plants, it needs to be dissolved by bacteria. Phosphate solubilizing bacteria are known to be able to convert insoluble phosphate into its soluble form (Mursyida *et al.*, 2015).

Phosphate solubilizing bacteria are bacteria that can produce a phosphatase enzyme that can convert insoluble phosphate elements into their dissolved forms (Gyaneshwar *et al.*, 2002). The availability of organic phosphate depends on microbe-enzyme interactions. Phosphate solubilizing microbes possess the ability to produce phosphatase enzymes that will be secreted around the rhizosphere of plants. These enzymes can break down bound-P compounds into soluble minerals. Phosphate solubilizing bacteria also release organic acids, which play a key role in the release of P from Al, Fe, and Ca cations in the soil (Joner *et al.*, 2000). The

*Corresponding author

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main mechanism of phosphate solvent is engaged in the production of organic acids or enzymes that support organic phosphate mineralization in the soil (Mubarik *et al.*, 2016).

For decades, chemical fertilizers have been utilized for the fulfillment of plant nutrients even though they bring harms to the underground flora, fauna, and even microorganisms. The accumulation of these environmentally unfriendly substances will eventually lead to the reduction of soil fertility. The long-term use of chemical fertilizers also brings a negative impact on the environment (Kochakinezhad *et al.*, 2012; Lynn *et al.*, 2013). Therefore, the provision of biological fertilizers containing living microorganisms can be one of the best solutions to improve soil quality (Mahanty *et al.*, 2017). Compared to chemical fertilizers, biological fertilizers are more effective in increasing soil nutrients and minerals. Biofertilizers do not leave residual effects on the soil (Vessey, 2003; Kumar, 2014). Thus, the administration of phosphate solubilizing bacteria as a biological fertilizer can be beneficial, as they can provide the nutrients needed by plants and increase the productivity of the plants (Mohammadi and Sohrabi, 2012).

Phosphate solubilizing bacteria's predominant roles in agriculture include spurring plant growth, decomposing organic matter, supporting the nutrient cycle, and reducing the use of chemical fertilizers (Desire *et al.*, 2018). *Bacillus* sp. strain AZ17 and *Pseudomonas* sp. strain AZ5 found in the rhizosphere of a plant were reported to have the potential to dissolve phosphate (Zaheer *et al*., 2019). The discovery of *Serratia marcescens* as a commercial inoculant and potential as biological fertilizer and reduce chemical fertilizer (Astriani *et al.*, 2016). Another previous study, such as Rosales *et al.* (2017) has successfully identified several *Simmondsia chinensis* endophytic bacteria such as *Bacillus* sp., *Methylobacterium aminovorans*, *Oceanobacillus kimchi*, *Rhodococcus pyrinivorans*, and *Streptomyces sp.*, as phosphate solubilizing bacteria which normally colonize the roots of a plant. Other bacteria that demonstrate phosphate solubilization capacities, such as *Pseudomonas oryzihabitans*, *Pseudomonas psychrotolerans*, *Stenotrophomonas maltophila*, *Bacillus megaterium*, and *Acinetobacter baumannii*, dwell in limestone soils (Fitriyanti *et al.*, 2017).

Several studies have isolated phosphate solubilizing bacteria from soil, the rhizosphere of plants, and limestone, but only a few publications have discussed the superiority of microbiomes obtained from indigenous rumen in mineralizing phosphate. Indigenous microorganisms (IMO) are a great technology for maintaining underground flora and fauna, as well as protecting microorganisms in the soil (Kumar and Gopal, 2015). In addition, Zamudio *et al.* (2001) discovered indigenous cow rumen as a source of phytase that was able to catalyze phytate (myo-inositol hexakisphosphate) into inorganic orthophosphate and a series of low phosphoric acids such as monophosphate in the digestive tract of ruminants. Based on the information, the IMO of cow rumen may contain a great number of superior microbes. Therefore, this research aimed to isolate phosphate solubilizing bacteria from a typical sample of indigenous cow rumen and thus provide novel information about the potentials of indigenous rumen found in East Java, Indonesia. This research is hopefully able to contribute to the future development of eco-friendly biofertilizer, where soil fertility can be refined through the application of phosphate solubilizing microbes.

MATERIALS AND METHODS

Materials

The indigenous microorganisms (IMO) samples were obtained from 10 kg of cow rumen; mixed with 20 L of coconut water; 20 L of rice water; 3 L of sugarcane water; 50 g of crushed dried shrimp. Coconut water and dried shrimp as the nitrogen (N) and mineral source; rice water, and sugarcane water as the carbon (C) source were used as the microbe growing medium. All the ingredients were poured into a 200 L plastic drum, then homogenized it and tightly shut. An aerator was installed on the drum. The IMO fermentation process lasted for 15 days of incubation (modified from Manullang and Rihab, 2018). Pikovskaya medium contained 10 g of glucose, 5 g of $Ca_3(PO4)_2$, 0.5 g of $(NH_4)_2SO_4$, 0.2 g of NaCl, 0.1 g of $MgSO_4 \cdot 7H_2O$, 0.2 g of KCl, 0.5 g of yeast extract, 0.002 g of $MnSO_4 \cdot H_2O$ and 0.002 g of $FeSO_4 \cdot 7H_2O$ (Nautiyal, 1999).

Indigenous microorganisms (IMO) sample collection

The IMO of the rumen were collected from Gogodeso village in Blitar, East Java, Indonesia. Observation of IMO was conducted on a daily basis until the mixture was ripe, indicated by the absence of smell, white coating on the surface, and color change from brown to dark brown (Manullang and Rihab, 2018). The IMO samples were collected using a purposive sampling method. Five samples were obtained from three random sampling points. These samples were collected five days after fermentation at a depth of 5-10 cm from the liquid surface.

Isolation of phosphate solubilizing bacteria

Phosphate solubilizing bacterial isolation was performed using dilution method and pour plate on a solid Pikovskaya medium with the addition of 0.2% agar composition. One gram of sample was inserted into multilevel physiological saline (from 10^{-1} until 10^{-6} dilution). At 10^{-4} until 10^{-6} dilution, 0.1 mL of sample was taken in duplicate before it was inoculated on the Pikovskaya medium (Wang *et al.*, 2017). It was then incubated for three days. Clear zones (halo zone) formed around the bacterial colonies indicated the bacteria's ability to dissolve phosphorus (P). The colonies were transferred for purification until a single colony was obtained. Then, the shape, margin or edge, elevation, and the color of the colony were observed (Benson, 2001).

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Qualitative estimation of phosphate solubilizing activity

The purified bacterial isolates were inoculated on a solid Pikovskaya medium. The isolates were dripped on the medium and incubated for seven days at room temperature (Jesus *et al.*, 2017; Zhang *et al*., 2017). Clear zones formed by the isolates were marked, and the Solubility Index (SI) of each colony was measured using the formula below (Mursyida *et al.*, 2015) :

Solubility index (SI) = [Diameter of clear zone (mm) – Diameter of the colony (mm)]/ Diameter of the colony (mm)

Quantitative estimation of phosphate solubilizing activity

One loop of the selected bacterial isolate was inoculated in 50 mL of liquid Pikovskaya, then incubated in a shaking incubator for 48 h. After the incubation process, 1 mL of culture was transferred into 100 mL of liquid Pikovskaya and incubated. Every 24 h, 1.5 mL of culture was collected and centrifuged to separate the supernatant. One milliliter of supernatant was reacted with color-forming reagent (2.5 mL of 2.5% sodium molybdate and 1 mL of 0.3% hydrazine sulfate) in duplicate, and heated in the water bath for 10 min. After the color of the solution was changed to blue, phosphate solubilizing activity was measured using a Genesys 20 spectrophotometer at 830 nm. The solubilized phosphate concentration (mg /L) was derived from the standard curve equation generated by dissolving KH_2PO_4 (Lynn *et al.*, 2013; Ghao *et al.*, 2016; Sanjotha and Manawadi, 2016).

Hypersensitivity assay

Hypersensitivity assay was performed to identify bacteria response to tobacco (*Nicotiana tabaccum* L.) leaf. In brief, 1 mL of bacterial isolate (cell density 10^8 CFU/mL) was injected on the below surface of a tobacco leaf by using a syringe (Jiang *et al.*, 2016). The injection of bacterial suspension was conducted in triplicate, quadruplicate, and pentuiplicate. Hypersensitivity response was observed at 24 h, 48 h, and 72 h after inoculation time. The leaf that was injected with sterile media was used as a negative control and pathogenic bacteria (*Pseudomonas syringae*) was used as positive control as shown by necrotic symptoms around the suspension injection area. Isolates that showed the symptoms were considered as pathogenic and were excluded from further testing (Abdallah *et al.*, 2016).

Assay for antagonism within isolate

The selected isolates were grown in Nutrient Broth medium for 24 h. Each 400 µL of culture was inserted into 40 mL of semisolid nutrient agar. Wells were formed using a sterile straw, creating rooms for testing other isolates. Each well was filled with 15-20 µL of culture tested. Sterile aqua dest was used as a negative control, while kanamycin (1 mg/mL) was used as a positive control. The cultures were incubated at the room temperature for 24 h (Fitriyanti *et al*., 2017). Antagonist isolates were characterized by inhibitor zones formed as in positive control. Isolates that showed inhibitor zones were not further processed as biofertilizer candidates.

Identification of bacterial isolate based on 16S rRNA gene

Isolation of genomic DNA

The genomic DNA was isolated as described by Sambrook and Russel (2001), with some modification. Briefly, the selected bacterial isolates were grown in nutrient broth for 24 h. Then,1.5 mL bacterial culture was transferred into 1.5 mL microcentrifuge tube and. centrifuged at 8000 rpm for 10 min. The supernatant was then removed, while the pellet was washed with STE buffer (composition: 0.3 M sucrose; 25 mM Tris-HCl; 25 mM EDTA.2Na pH 8) then centrifuged at 8000 rpm for 10 min. Pellets are washed 3 times repeatedly. Subsequently the supernatant was removed and the pellets obtained were added 200 µL STE buffer and 45 µL lysozyme (20 mg/mL) slowly turned and then incubated at 55 °C for 1 h to form a protoplast. Proteinase-K (20 µL) was added into the concoction. After that, it was incubated at 55 °C for 60 min. CTAB 10% (400 µL) was mixed with 0.7 M NaCl. The solution was added with 1× phenol: chloroform (25:24) and centrifuged at 12000 rpm for 10 min. The clear phase was transferred into a new tube and subsequently, 0.6× isopropanol volume and 20 µL of sodium acetate and was added . Next, the supernatant was removed, and the pellet was washed with 1 mL of 70% ethanol. The DNA was then collected and dried for 1 h. Finally, the DNA was dissolved in 50 µL of sterile nuclease free water. The isolated DNA was stored at 4 °C.

Amplification of bacterial isolates 16S rRNA gene and analysis of the phylogenetic tree

The extracted DNA was amplified by Polymerase chain reaction (PCR) using primer 63F (5'-CAG GCC TAA CAC ATG CAA GTC-3') and 1387R (5'-GGG CGG WGT GTA CAA GGC-3') (Marchesi *et al.*, 1998). PCR conditions used consisted of pre-denaturation (94 °C, 4 min), denaturation (94 °C, 45 sec), annealing (55 °C, 1 min), elongation (72 °C, 1 min 10 sec), and post PCR (72 °C, 7 min) for 30 cycles. The DNA was separated using mini-gel electrophoresis with 1% agarose (75-volt electricity for 45 min). DNA visualization was conducted on a UV transilluminator using ethidium bromide staining.

The DNA sample was then sent for sequencing service. The result of the DNA sequencing was trimmed and assembled using ChromasPro version 1.5. Data were BLAST with genome data from website NCBI/National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST). The data were re-

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analyzed by aligning the sequence using MEGA 6.1 (Tamura *et al.*, 2011). Phylogenetic tree describes the relationship of isolate MTA1 and other bacteria in the ingroup clade or the outer group clade. The construction of the phylogenetic tree was based on the Neighbor-Joining Tree method with 1000× Bootstrap repetitions.

RESULTS

Isolation and determination of phosphate solubilizing bacteria

Figure 1 shows some bacteria formed clear zones around the colonies and some bacterial colonies did not formed the clear zones. Bacteria that formed clear zones were marked as phosphate solubilizing bacteria. Seven bacterial isolates were found to form clear zones around the colonies. They are SMAD1, SMAD2, SMAD3, SMAD4, MTA1, MTA2 and MTA3.

The selected bacteria were purified on a Pikovskaya medium for qualitative estimation of phosphate solubilizing activity by observing a clear zone around the bacterial colonies. Phosphate solubilization index (SI) was determined as the basis to identify phosphate solubility bacteria. Four isolates (SMAD1, SMAD2, SMAD3, MTA1) were found able to form clear zones with solubilization index (SI) ranging between 4.71 and 7.66 mm (Figure 2).

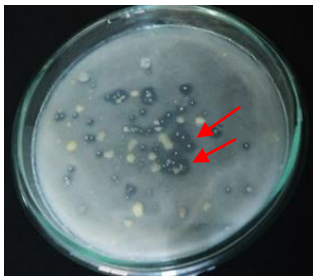

Figure 1: Phosphate solubilizing bacterial isolates on Pikovskaya Medium. Red arrows: colony forming a clear zone.

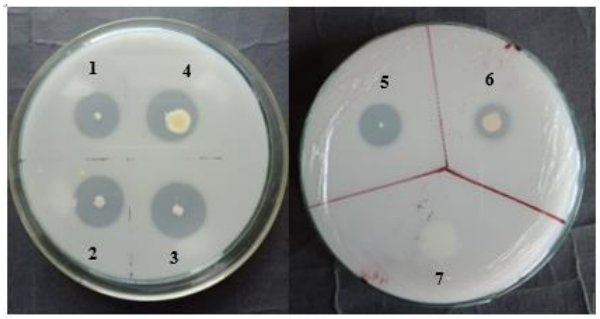

Figure 2: Solubilization index (SI) of bacterial isolates on Pikovskaya medium after 7 days of incubation. (1) SMAD1: 4.71 mm, (2) SMAD2: 5 mm, (3) SMAD3: 5.4 mm, (4) SMAD4: 3.5 mm, (5) MTA1: 7.66 mm, (6) MTA2: 0.83 mm, (7) MTA3: 0 mm.

Quantitative estimation of phosphate solubilizing activity

The four isolates, namely SMAD1, SMAD2, SMAD3, and MTA1, were chosen due to their high solubilization index as indicated clear zone (halo zone) formation. Isolate MTA1 reported an optimum concentration of solubilized phosphate compared to the other isolates in a Pikovskaya liquid medium. The highest concentration of solubilized phosphate (up to 278.42 mg/L) was observed on day 5.

To estimate phosphate solubilizing activity, then a curve was made from day 0 to day 6 (Figure 3). Based on the curve, isolates SMAD3 and SMAD1 were observed to have the second-highest phosphate solubilizing activity after isolate MTA1 (111.75 mg/L on day 3 and 103.12 mg/L on day 4). The phosphate solubilizing activity of isolate SMAD2 was observed on day 3, with a concentration of 69.93 mg/L.

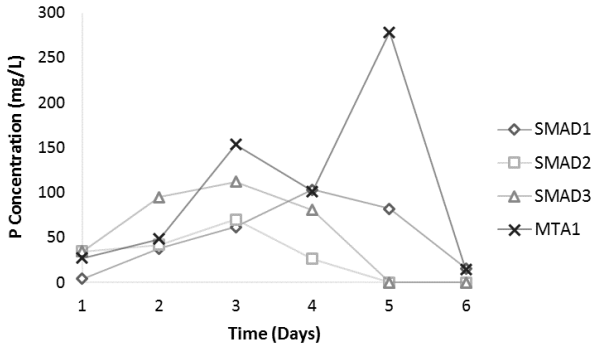

Figure 3: The concentration of solubilized P after 6 days of observation on the selected isolates.

Hypersensitivity assay

Nicotiana tabacum L. leaves were injected with four bacterial isolates (SMAD1, SMAD2, SMAD3, MTA1); no necrosis sign was observed on the leaves until five days of treatment (Figure 4c-f). Necrotic changes are shown in Figure 4b, where brown spots are found around the area of the leaf after being injected with *Pseudomonas syringae,* a pathogenic bacterium. The reaction of the four isolates was similar to that of sterile aqua dest that showed an absence of resistance response to pathogens (Figure 4a). The result of the four-isolate testing was hypersensitivity negative and eco-friendly.

Assay for antagonism within isolate

Qualitative and quantitative estimation of the bacterial phosphate solubilizing activity had been conducted, and the result of the hypersensitivity assay had demonstrated negative symptoms on a tobacco leaf. Therefore, the next stage was to test the antagonism of these isolates against one another and to compare it with positive control using kanamycin. In Figure 5, it is apparent that isolate MTA1 showed no inhibitor area around other isolates (SMAD1,

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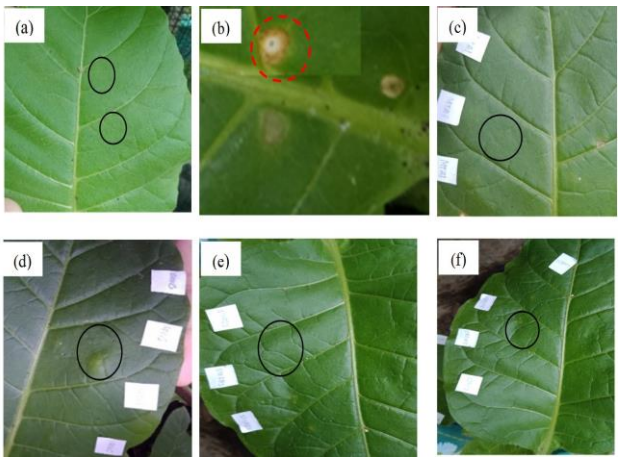

Figure 4: Hypersensitivity assay of bacterial isolates on *Nicotiana tabacum* L. leaf showing brownish blotches indicated as necrotic sign. (a) sterile media (negative control) (b) pathogenic *Pseudomonas syringae* (positive control) (c) MTA1 (d) SMAD1 (e) SMAD2 (f) SMAD3.

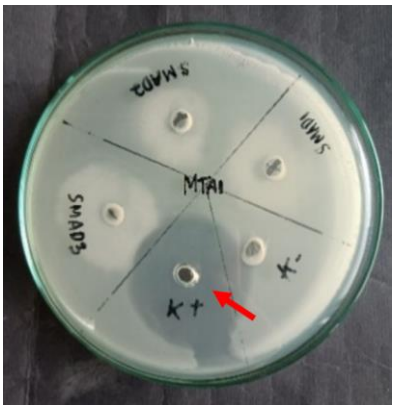

Figure 5: Antagonist test of MTA1 isolates against other isolates on NA semisolid media. Red arrow: positive control kanamycin (1 mg/mL) and negative conrol (sterile aquadest).

SMAD2, SMAD3). The reaction of isolate MTA1 is similar to that of negative control using aqua dest, where no inhibitor zones were formed. On the other hand, clear zones that were formed in the kanamycin wells (indicated by red arrow in Figure 5) showed inhibition reactions towards the growth of other bacterial isolates. This finding suggests that isolate MTA1 has no inhibitory potency towards the growth of other bacteria and therefore, this bacterial isolate can be used as a candidate for eco-friendly biofertilizer.

Bacterial identification

The four selected isolates shared similar characters of the colony based on shape, margin, elevation, and color (Table 1). These isolates were found to have round (circular) shape, smooth margin, flat elevation, and white and cream color. Based on Gram staining, isolates SMAD1, SMAD2, SMAD3, and MTA1 were categorized into Gram-positive. The cell shape of all isolates was rods (Figure 6). In addition, the result of the biochemical testing using Microbact, positive results in motility, nitrate, glucose reducing, sucrose, and catalase (Table 2). Based on the colony and cell shape, Gram staining, and biochemical testing, one of the isolates was selected for identification. Due to its high phosphate solubilizing activity, isolate MTA1 was selected for molecular identification.

The amplification of the 16S rRNA gene of isolate MTA1 was conducted using primer 63F (5'-CAG GCC TAA CAC ATG CAA GTC-3') and primer 1387R (5'-GGG CGG WGT GTA CAA GGC-3'). The amplification result produced a 1300 bp band as shown in Figure 7. Based on the 16S rRNA gene sequence analysis (Table 3) and the construction of a phylogenetic tree, isolate MTA1 showed 99% sequence homology with *L. plantarum* (Figure 8).

DISCUSSION

Seven phosphate solubilizing bacteria were successfully isolated from the indigenous microorganisms (IMO) of cow rumen. The fermented rumen IMO solution is composed of macro and micronutrients, as well as beneficial microbial communities or phyto-regulators, which indirectly have an impact on plant growth, leaf formation, root strength, seed germination, pest control, and plant disease prevention (Wang and McAllister, 2002). Phosphate is a key element for soil fertility and plant productivity. Therefore, since the availability of phosphate is limited in the soil, the discovery of the seven phosphate solubilizing bacteria isolated from the rumen IMO is highly beneficial and potential in the field of biofertilizer technology. The phosphate solubilizing bacteria obtained from the IMO of cow rumen were proven to have higher phosphate solubilization indices (4.71 to 7.66 mm) (Figure 2). The treatment conditions were maintained the same as those in Suleman *et al.* (2018) who showed phosphate solubilizing indices between 3.2 and 5.8 mm from rhizospheric bacteria, and between 0.125 and 2.375 mm from limestone quarry bacteria (Fitriyanti *et al.*, 2017). The solubilization index of rhizobacteria range from 0.125 to 3.5 mm (Astriani *et al.*, 2020).

The estimation of phosphate solubilizing activity (Figure 3) also showed that the isolates found in this study had a higher concentration of solubilized phosphate in a Pikovskaya liquid medium (69.93 mg/L to 278.42 mg/L). Over the last five years, the highest phosphate solubilization index (SI) (1.00 mm) and quantitative estimation of phosphate (80.61 mg/L) was found in isolate QC3 (Mursyida *et al.*, 2015). Cao *et al.* (2018) also reported a high phosphate solubilization index in a liquid medium of rhizobacteria (125.88 mg/L) within 10 day incubation. Besides, Aarab *et al.* (2013) discovered that rhizobacteria were able to maximum phosphate solubility were observed 298.66 mg/L after seven days of

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Table 1: Characteristics of selected isolates growth.

No	Isolate code	Colony characteristics				Gram	Diameter of colony (mm)
		Shape	Color	Margin	Elevation		
1	SMAD1	round (circular)	white	smooth	flat	+	0.35
2	SMAD2	round (circular)	cream	smooth	flat	+	0.41
3	SMAD3	round (circular)	white	smooth	flat	+	0.38
4	MTA1	round (circular)	white	smooth	flat	+	0.39

Table 2: The result of the biochemical testing.

Parameter	SMAD1	SMAD2	SMAD3	MTA1
Motility	motile	motile	motile	motile
Oxidase	negative	negative	negative	negative
Catalase	positive	positive	positive	positive
Indole production	negative	negative	negative	negative
TSIA test	Alk/Alk, G-H2S	Alk/AsH2S	Alk/AsH2S	Alk/Alk, G-H2S
Voges Proskauer	negative	negative	negative	negative
Spore	negative	negative	negative	negative
Nitrate	positive	positive	positive	positive
Glucose	positive	positive	positive	positive
Xylose	negative	negative	negative	negative
Citric	negative	negative	negative	negative
Sucrose	positive	positive	positive	positive
Urease	negative	negative	negative	negative
Malonate	negative	negative	negative	negative

Table 3: 16S rRNA gene sequence of isolate MTA1 aligned with NCBI (BLASTX) data.

Description	Max score	Total score	Quary cover	E value	Identity	Access number
Lactobacillus plantarum strain L15 16S ribosomal RNA gene, partial sequence	2043	2043	100%	0.0	99.47%	MK713565.1
Lactobacillus pentosus strain CO1 16S ribosomal RNA gene, partial sequence	2043	2043	100%	0.0	99.47%	MK689398.1
Lactobacillus plantarum strain CB5 16S ribosomal RNA gene, partial sequence	2043	2043	100%	0.0	99.47%	MK687387.1
Lactobacillus plantarum strain LVP40 16S ribosomal RNA gene, partial sequence	2043	2043	100%	0.0	99.47%	MK676008.1
Lactobacillus sp. strain LB51 16S ribosomal RNA gene, partial sequence	2043	2043	100%	0.0	99.47%	MK616557.1

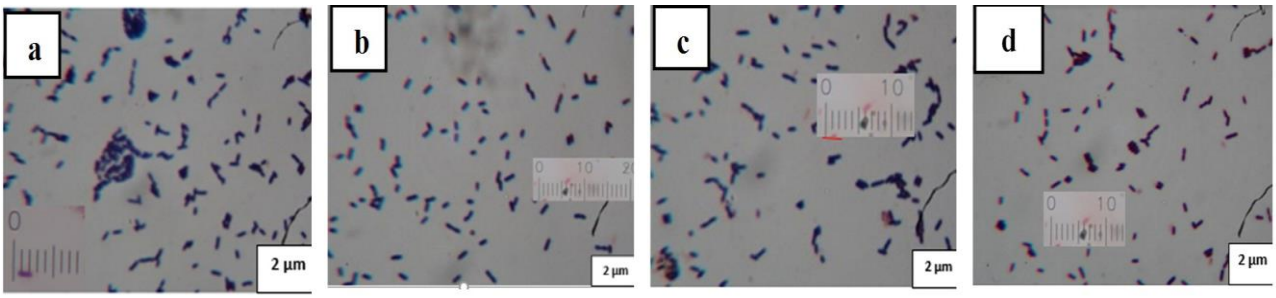

Figure 6: Gram-staining of the selected isolates at 1000× magnification. (a) SMAD1, (b) SMAD2, (c) SMAD3, (d) MTA1. The micrometer scale line had a measurement of 2 µm.

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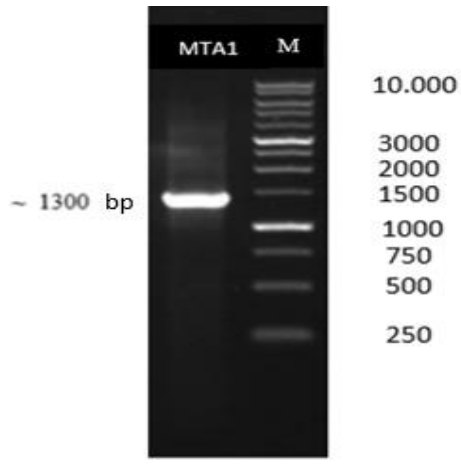

Figure 7: Visualization of PCR amplification of 16S rRNA gene on agarose 1%. M: 1 kb marker ladder; MTA1: PCR product of bacterial sample.

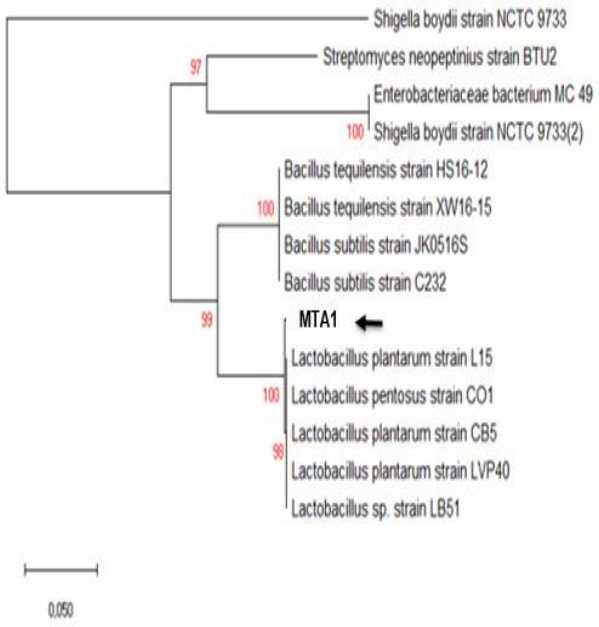

Figure 8: Phylogenetic tree construction of isolates was selected based on 16S rRNA gene sequence.

incubation. In another relevant study, the highest concentration of solubilized phosphate observed was 134 mg/L, while the lowest was 38 mg/L (Ruangsanka, 2014). Astriani *et al.* (2020) reported the highest phosphate solubilizing activity on day 3 and day 5 were 75.39 and 74.33 mg/L in liquid culture, respectively.

The highest phosphate content (278.42 mg/L) was found in isolate MTA1, followed by SMAD1 (111.75 mg/L), SMAD3 (103.12 mg/L), and SMAD2 (69.93 mg/L). The rate of soluble phosphate uptake decreased after five days on isolates SMAD1, SMAD2, SMAD3, and after six days on isolate MTA1 (Figure 3). The decreasing of uptake phosphate happened because of the reduction of the bacterial population and cell metabolism activity (Mursyida *et al*., 2015). The increase in the ratio of clear zones and solubilized phosphate index due to the release of organic acid by bacteria. Variation in phosphate solubilization index is dependent on the ability of bacteria in secreting organic acids to dissolve phosphate (Tripti *et al.*, 2012). Phosphate solubilization occurs due to proton release, such as H^+ released by tricalcium phosphate through the mechanism of $Ca_3(PO_4)_2 + 2H^+ \rightarrow 2CaHPO_4 + Ca^{2+}$ in a Pikovskaya medium. This reaction occurs due to the ability of the phosphate solubilizing microbes to produce a phosphatase enzyme that can be secreted to the environment. The activity of the enzyme can trigger the mineralization of organic phosphorus compounds (Joner *et al.*, 2000; Yu *et al*., 2014).

The selected four isolates showed no pathogenic reaction on *Nicotiana tabaccum L*. leaf, so that they can be considered for biofertilizer candidates (Abdallah *et al*., 2016). Also, these isolates did not demonstrate any inhibitory activity against one another. The phosphate solubilizing bacteria that have been tested in this study thus fulfill the criteria of eco-friendly biofertilizer that can be used in crops. According to Bashan *et al.* (2014), environmentally friendly bio inoculants for biofertilizers do not show necrotic symptoms in plants nor inhibit the growth of other isolates when formulated. Lynn *et al.* (2013) added that biofertilizers from a mixture of several strains of bacteria are more effective in accelerating the growth of plants.

The morphological characteristics of the inoculated bacteria are similar. They all have a round shape, smooth margin, flat elevation, and are categorized into Gram-positive rods. Isolate MTA1 showed the highest phosphate solubilization index compared to the other isolates, therefore this isolate was selected for further molecular identification. Phylogenetic analysis indicated that isolate MTA1 was closely related to *Lactobacillus plantarum* with similarity level of 99%. *Lactobacillus plantarum* is a versatile Gram-positive bacterium commonly found in the gastrointestinal tract of ruminants (Nicoloff *et al.*, 2004). *Lactobacillus plantarum*, as the main component of effective commercial microorganisms, can increase plant growth and crop harvest (Javaid and Mahmood, 2010). *Lactobacillus plantarum* STO3 from the guano of bat cave was reported to be able to dissolve phosphate (SI = 2.23) and synthesize indole acetic acid (1.88 ppm) (Suryanto *et al.*, 2017). Besides, *L. plantarum* was also reported as one of the lactic acid bacteria which can perform high phytase activity (Zamudio *et al.*, 2001), utilize phosphate sources for P mineralization, and have an antimicrobial activity that positively affects phosphate solubilization (Suryanto *et al.*, 2017).

The finding of this study is highly important because *L. plantarum* has been proven as a cow rumen bacterium with the highest phosphate solubilizing activity and this finding has never been reported in the previous research.

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Phosphate solubilizing bacteria from IMO of cow rumen can also help convert organic phosphate into its soluble form as a plant nutrient through phosphate mineralization. Since the current study was merely focused on isolating phosphate solubilizing bacteria, an investigation of the effect of phosphate solubilizing bacteria in a single form or consortium on plant growth and production needs to be conducted in the future. It is also necessary to consider conducting a series of field studies to discover more effective, efficient, and environmentally friendly or eco-friendly biofertilizer that are useful to fulfill plant nutrition and reduce the use of chemical fertilizers.

CONCLUSION

Four phosphate solubilizing bacteria, namely SMAD1, SMAD2, SMAD3, MTA1, were successfully isolated from the indigenous microorganisms (IMO) of cow rumen in Blitar, East Java. Clear zones were observed around the bacterial colonies (solubilizing index spanned from 4.71 to 7.66 mm), and the bacteria phosphate solubilizing activity was found between 69.93 and 278.42 mg/L. Isolate MTA1 had the highest phosphate solubilization index and solubilized phosphate activity (7.66 mm and 278.42 mg/L, respectively). Based on the 16S rRNA gene analysis, isolate MTA1 showed 99% sequence homology with *L. plantarum*. The discovery of *L. plantarum* in this study and provides an insight that cow rumen contains bacteria that have extremely high phosphate solubilizing activity. Phosphate solubilizing bacteria isolated in this study can be used for the future development of effective, efficient, and eco-friendly biofertilizer and bio inoculants to minimize the use of synthetic chemical fertilizers.

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