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Exploration of phosphate solubilizing bacteria from mangrove soil of Lamongan, East Java, Indonesia

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Abstract. *Fatimah, Aula N, Salsabila S, Ramly ZA, Rose SY, Surtiningsih T, Nurhariyati T. 2023. Exploration of phosphate solubilizing bacteria from mangrove soil of Lamongan, East Java, Indonesia. Biodiversitas 24: 1272-1278.* The objective of this research was to obtain and characterize bacterial isolates from mangrove soil Lamongan, East Java, Indonesia, screen the ability of Phosphate Solubilizing Bacteria (PSB) and determine their Phosphate Solubilization Index (PSI), and identify the species of most potential PSB. Bacteria were isolated and characterized macroscopically on Nutrient Agar media. Microscopic characteristics were observed by Gram staining. Phosphate solubility test carried out using Pikovskaya Agar media. The highest PSI value bacteria was identified molecularly using 16S rRNA gene. This explorative study was analyzed descriptively. The results showed that a total of 17 isolates were obtained, of which 14 isolates were classified as Gram-positive bacteria and 3 isolates were classified as Gram-negative bacteria. All the 17 bacterial isolates showed different colony morphology. Amongst the 17 isolates, 12 of them were PSB. The TL-8 isolate was the most potential PSB, with a PSI value of 2.82. Based on the results of molecular identification with the 16S rRNA gene, TL-8 was identified as *Lysinibacillus fusiformis* TL-8, had 99.57% similarity with *L. fusiformis* strain NBRC 15717T.102 with a query cover of 100%.

Keywords: 16S rRNA gene, *Lysinibacillus fusiformis*, mangrove, phosphate solubilizing bacteria

INTRODUCTION

Phosphorus (P) has an important role in helping plant growth, where P will be mobile in plants so that it is easy to move from old leaves to other growing points in plants. P has many roles in plants, including one of the constituent elements of ATP compounds needed for the energy transfer process, one of the constituent elements of NADP compounds needed for photosynthesis, and one of the constituent elements of cell membranes (phospholipids) (Yahra 2020). P deficiency in plants has been studied to cause a significant reduction in the rate of photosynthesis in several food crops such as citrus leaves, rice, wheat, white lupin, wheat, and *Zizania latifolia* (Liu et al. 2015; Ansari et al. 2016; Mahajan et al. 2017; Li et al. 2018; Long et al. 2019; Kour et al. 2020; Xu et al. 2020).

Chemical fertilizers are widely used in the agricultural and plantation sectors as an effort to increase the P element for plants. However, excessive use of chemical fertilizers in the long term can cause an imbalance of nutrients in the soil. To overcome this problem, many researchers have developed biofertilizers. Applying biofertilizer to rhizosphere soil does not aim to add soil nutrients, but to promote availability of nutrients for plants, with the help of microorganisms. Biofertilizer is a biological fertilizer that contains microbes that promote plant growth. Microbes in biofertilizers can consist of only one type or can also be a combination of several types of microbes, called a microbial consortium. The combination of several types of soil bacteria that are used together appropriately in the application of biofertilizers in organic farming systems can have a positive impact on the availability of nutrients needed by plants, can control pests and diseases in plants, and increase plant growth and productivity (De-Bashan et al. 2012; Bruggen 2016).

One of the microbial consortium's components in the biofertilizer is Phosphate Solubilizing Bacteria (PSB). PSB has an important role in overcoming the limited availability of P for plants, where 75-90% P cannot be utilized by plants because it was bound by metal cation complexes such as Al, Ca, and Fe which makes P immobile in the soil (Sharma et al. 2013). PSB is able to produce organic acid compounds, where this organic acid will bind metal cations that were originally bound to P elements. Therefore, P elements can be separated from bonds with cations so that P elements become available to plants.

Mangrove forest is one of the ecoton ecosystems that are often found on the coast of the sea in areas with tropical and subtropical climates in the world (Liu 2014). Indonesia as a tropical country has the largest mangrove forest area in the world with a total area of three million hectares of mangrove forest, representing 23% of the global mangrove ecosystem (Hapsari et al. 2020). One of the vast natural mangrove forest areas located along the coast as well as the river estuary is located in the coastal area of Lamongan. Lamongan is a city located in the province of East Java, Indonesia and has a coastline of approximately 47 km, which borders the Java Sea, Indonesia (Merkel 2012). A

fairly high P content is commonly found in mangrove sediments, such as in the Wonorejo Surabaya Ecotourism mangrove area, Indonesia with a P nutrient content of 0.038 ppm. From the results of PSB exploration in the Wonorejo Ecotourism mangrove area in Surabaya, 12 PSB isolates were obtained, which were dominated by the *Bacillus* genus (Pradipta 2016; Supriyantini 2018). In previous study, Fatimah et al. (2021) had successfully isolated 19 isolates PSB from Mangrove soils in Jenu Tuban. Several researchers had isolated molds and yeast as phosphate solubilizer from mangrove rhizosphere in Jenu Tuban (Nurhariyati et al. 2020; Surtiningsih et al. 2020). This proves that PSB is present in the mangrove rhizosphere to help mangrove plants obtain P elements.

Phosphate Solubilization Index (PSI) is an important value to measure the ability of PSB to dissolve phosphate. Identification of bacterial isolates can be done by conventional methods, such as macroscopic and microscopic identification as well as molecularly. Molecular identification of bacteria can be done by sequencing using the 16S rRNA gene. In the 16S rRNA gene, hypervariable regions are found which can facilitate the process of identifying the type of bacteria (Ricci et al. 2020). Thus, the aim of this study was to isolate and identify phosphate solubilizing bacteria from the mangrove rhizosphere area of Kutang Beach, Lamongan, East Java, Indonesia. The PSB species that had the highest phosphate solubilization index value could become a potentially biofertilizer candidates.

MATERIALS AND METHODS

This research was conducted exploratory. The materials used in this study were ten soil samples taken from ten spots in the mangrove rhizosphere area of Kutang Lamongan Beach, East Java, Indonesia.

Isolation of mangrove soil bacteria

A total of ten soil samples were taken from ten spots in the mangrove rhizosphere area of Kutang Lamongan Beach, East Java, Indonesia.

Each sample was weighed as much as 25 g then dissolved 225 mL of sterile distilled water and homogenized using a shaker for 30 minutes. Serial dilutions were made from 10^{-1} to 10^{-7} by transferring 1 mL of the suspension to a new test tube containing 9 mL of distilled water. Bacterial isolation was carried out by taking 1 mL of mangrove soil samples suspension from the desired dilutions tubes (10^{-1}, 10^{-4}, and 10^{-7}) then were grown on NA medium and incubated at 30°C for 24-48 hours.

Bacterial purification

Single colonies with different characters were transferred to a new Petri dish containing sterile NA media by streak method and repeated until only one type of colony on NA media was obtained. The purified bacterial colonies were then transferred to NA slant media using streak method and incubated at 30°C. Single bacterial colonies on NA media were identified by visual observation of the colony morphological characteristics in the form of colony shape, colony edge, elevation, colony color, and colony surface. Colonies with different morphological characteristics were marked with different isolate codes. Then, bacterial isolates were characterized microscopically using the Gram stain test.

Phosphate solubility screening

Phosphate solubilizing bacteria screening was carried out on Pikovskaya medium and incubated at room temperature for 48-72 hours. The isolates that formed clear zones around the colonies were grown on Nutrient Broth (NB) media and incubated in incubator shaker at room temperature for 24 hours. The Optical Density (OD) of each culture isolate was measured with spectrophotometer at a wavelength of 600 nm. Bacterial cultures with an OD value of 0.5 were then dropped as much as 25 µL onto 6 mm blank paper discs, then placed on Pikovskaya Agar media with three replications and incubated for 72 hours at room temperature. The diameter of the clear zone and the diameter of the PSB colonies were measured with a caliper and then the Phosphate Solubility Index (PSI) was calculated using the following formula.

$$\text{PSI} = \frac{\text{mean of clear zone diametre (mm)}}{\text{mean of PSB colony diametre (mm)}}$$

DNA isolation

PSB with the highest phosphate solubilization index value was grown on NB media and incubated for 18 hours at room temperature then DNA isolation was carried out using Wizard Genomic DNA Purification Kit (Promega 2019).

PCR amplification

Amplification of the 16S rRNA gene of PSB isolate was carried out by PCR method using universal primers 27F (5' – AGAGTTTGATCMTGGCTCAG – 3') and 1429R (5′-GGTTACCTTGTTACGACTT-3′). PCR amplification was carried out for 35 cycles. Initial denaturation was carried out at 95°C for seven minutes. The denaturation of the DNA template was carried out at a temperature of 95°C for 30 seconds, then annealing at a temperature of 55°C for 45 seconds and continued with extension at a temperature of 72°C for one minute and 30 seconds, then the final extension was carried out at a temperature of 72°C for 7 minutes, then post-extension was carried out at 12°C. The amplification products were analyzed by electrophoresis on 1% agarose gels in TAE buffer with 1 L ethidium bromide and examined under UV transilluminator.

DNA sequence analysis

Determination of pure DNA sequence (sequencing) was done by sending purified DNA from the PCR product to PT. Genetika Science Tangerang, Banten, Indonesia then analyzed using Bioedit 7.04 software to obtain nitrogen base consensus and was analyzed using BLASTn program in NCBI.

Data analysis

The research data such as isolate characters, phosphate solubilization index, and the results of molecular identification of the most potent PSB isolates using the 16S rRNA gene were analyzed descriptively.

RESULTS AND DISCUSSION

Macroscopic and microscopic characteristics of bacterial isolates

The result showed that 17 bacterial isolates were obtained from ten samples of the mangrove rhizosphere area of Kutang Beach, Lamongan. All the 17 isolates had different macroscopic (colony) and microscopic characteristics on Nutrient Agar (NA) media (Figures 1 and 2). The morphological characteristics of isolates are presented in Table 1.

From Table 1, it can be seen that TL-3 had the most distinct macroscopic characteristics among other isolates. Colonies of TL-3 were yellow with a glisten or glossy texture. In terms of microscopic characteristics, there were only two isolates that had cocci or round cells.

Phosphate solubilization index

Seventy isolates that had passed the purification stage were tested for their potential to dissolve phosphate. An indication that bacteria were capable of dissolving phosphate was the formation of a clear zone around the colony. Of the seventy isolates, only twelve isolates from the mangrove rhizosphere area of Kutang Beach, Lamongan had phosphate solubilization potential (Figure 3). As a comparison, the negative control did not show any clear zone formation. Phosphate solubilization index data from 12 bacterial isolates are presented in Table 2. Of the 12 isolates tested for phosphate solubility, only the TL-8 isolate had the highest ability to dissolve $Ca_3(PO_4)_2$ contained in Pikovskaya Agar media with a phosphate solubility index of 2.82.

Molecular identification of most potent phosphate solubilizing bacteria with the gene encoding the 16S rRNA

The TL-8 isolate, which was the most potent isolate of PSB, was molecularly identified using the 16S rRNA gene to determine the species name. The results of DNA isolation from TL-8 isolate were amplified and visualized by agarose gel electrophoresis. The TL-8 DNA band was aligned with the 1,500 bp marker (Figure 4). The results of TL-8 16SrRNA gene sequencing were analyzed by BioEdit and matched to the Gene Bank database via BLASTn program at the NCBI site (Figure 5). Based on the analysis by BLASTn , the consensus sequence of isolate TL-8 had 99.57% similarity to *Lysinibacillus fusiformis* strain NBRC 15717T.102 with a query cover of 100%.

Table 2. Phosphate Solubilization Index (PSI) by phosphate solubilizing bacteria isolated from the mangrove rhizosphere area of Kutang Beach, Lamongan, East Java, Indonesia

Isolates code	Mean of PSB colony diameter (mm)	Mean of clear zone diameter (mm)	PSI
TL-3	6.58	9.33	1.42
TL-4	7.69	15.48	2.01
TL-5	7.33	9.36	1.28
TL-6	8.5	21.61	2.54
TL-7	6.82	10.38	1.52
TL-8	**7.87**	**22.17**	**2.82***
TL-11	8.55	21.82	2.55
TL-12	7.68	16.83	2.19
TL-13	11.56	25.86	2,24
TL-14	7.89	13.64	1,73
TL-15	7.8	21.6	2.77
TL-16	7.87	12.64	1.61

Note : *Most potential.

Table 1. Morphology Characteristics of bacteria from the mangrove rhizosphere area of Kutang Beach, Lamongan, East Java, Indonesia

Isolates code	Shape	Color	Texture	Margin	Elevation	Gram stain
TL-1	Circular	Cream	Dull	Entire	Flat	Bacilli +
TL-2	Circular	Cream	Rough	Entire	Flat	Bacilli -
TL-3	Circular	Yellow	Glisten	Entire	Convex	Bacilli +
TL-4	Circular	Milky	Dull	Undulate	Flat	Bacilli +
TL-5	Circular	Milky	Dull	Lobate	Flat	Bacilli +
TL-6	Punctiform	White	Dull	Entire	Flat	Cocci +
TL-7	Irreguler	Cream	Dull	Undulate	Flat	Bacilli +
TL-8	Irreguler	Cream	Dull	Lobate	Flat	Bacilli -
TL-9	Irreguler	Cream	Dull	Undulate	Convex	Bacilli -
TL-10	Irreguler	Cream	Rough	Filiform	Flat	Cocci +
TL-11	Circular	White	Dull	Filiform	Flat	Bacilli -
TL-12	Irreguler	Cream	Dull	Filiform	Flat	Bacilli +
TL-13	Irreguler	Cream	Wrinkle	Undulate	Pulvinate	Bacilli +
TL-14	Irreguler	Milky	Dull	Entire	Flat	Bacilli -
TL-15	Irreguler	Cream	Rough	Entire	Flat	Bacilli +
TL-16	Irreguler	Cream	Dull	Filiform	Flat	Bacilli +
TL-17	Circular	Cream	Dull	Filiform	Flat	Bacilli +

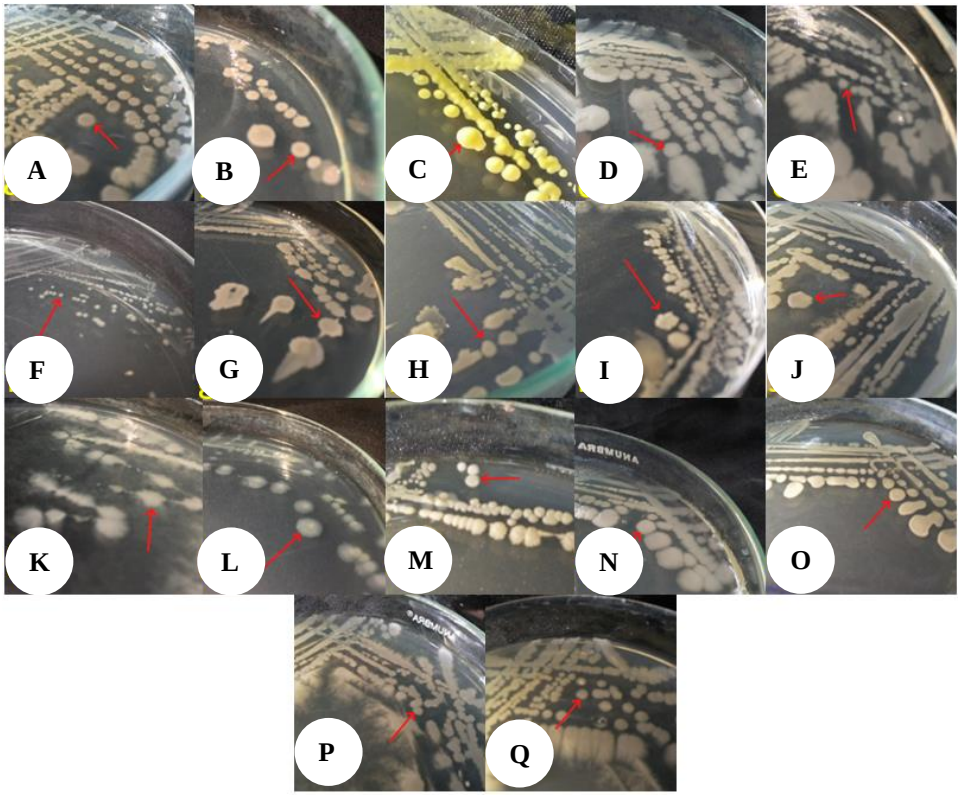

Figure 1. Colony morphology of isolated soil bacteria from Kutang Beach, Lamongan, East Java, Indonesia. Red arrows showed single colony of bacteria. A. TL-1; B. TL-2; C. TL-3; D. TL-4; E. TL-5; F. TL-6; G. TL-7; H. TL-8; I. TL-9; J. TL-10; K. TL-11; L. TL-12; M. TL-13; N. TL-14; O. TL-15; P. TL-16; Q. TL-17

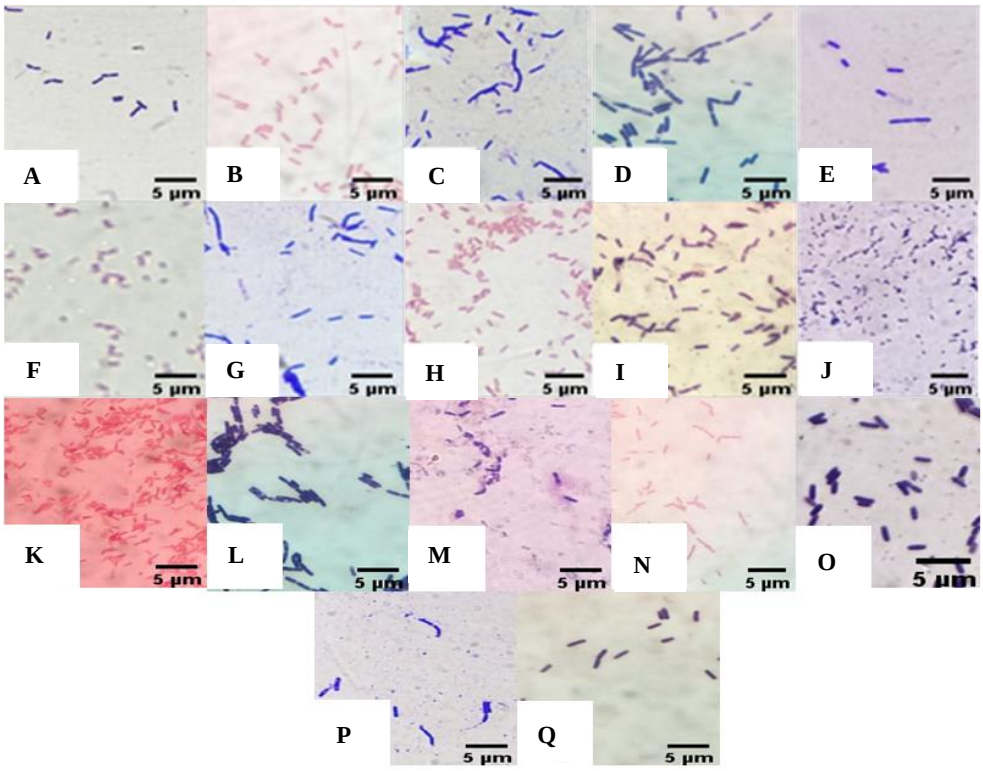

Figure 2. Morphology of bacteria cells isolated from the mangrove rhizosphere area of Kutang Beach, Lamongan, East Java, Indonesia. A. TL-1; B. TL-2; C. TL-3; D. TL-4; E. TL-5; F. TL-6; G. TL-7; H. TL-8; I. TL-9; J. TL-10; K. TL-11; L. TL-12; M. TL-13; N. TL-14; O. TL-15; P. TL-16; Q. TL-17

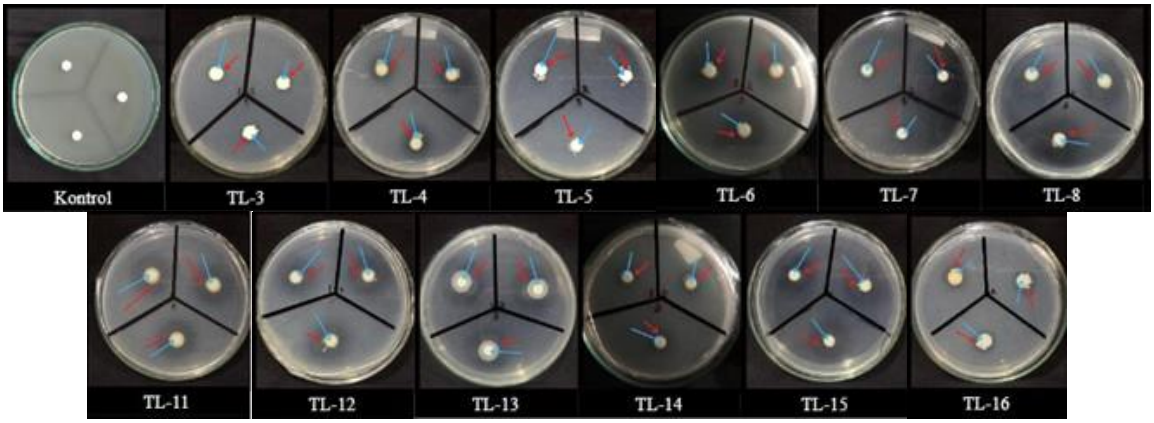

Figure 3. Results of phosphate solubility test of 12 bacterial isolates from the mangrove rhizosphere of Kutang Beach, Lamongan, East Java, Indonesia. Blue arrow (↗) pointed at PSB colony and red arrow (↗) pointed clear zone formation

Description

Lysinibacillus fusiformis strain NBRC 15717T.102 16S ribosomal RNA gene, partial sequence

Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Lysinibacillus fusiformis	2566	2566	100%	0.0	99.57%	1425	MN543804.1

Figure 5. Consensus sequence analysis results of 16SrRNA gene of TL-8 isolate (1405 bp) with BLASTn

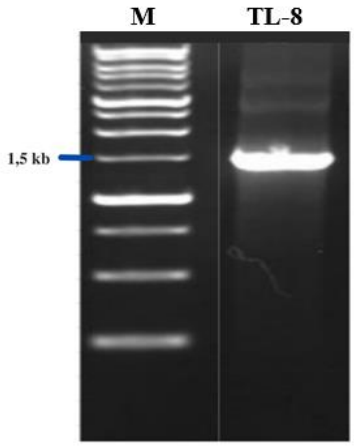

Figure 4. Electrophoregram of 16Sr RNA gene in 1% agarose gel: M= DNA ladder 1kb; TL-8 = 16SrRNA gene of TL-8 isolate (1500 bp)

Discussion

Phosphate Solubilizing Bacteria (PSB) are the main contributors of plant nutrition in agriculture and could play a pivotal role in making soluble phosphorus available to plants (Khan et al. 2010; Satyaprakash et al. 2017). This study aimed to isolate the bacteria from the mangrove rhizosphere area of Kutang Lamongan Beach, and to find out which isolates were capable of dissolving phosphate, to determine the value of the phosphate solubility index of each PSB, to determine the characteristics of PSB that have been isolated, and to determine the name of the PSB species by highest phosphate solubilization index value.

In this study, 17 bacterial isolates were isolated from the ten samples of mangrove rhizosphere soil of Kutang Beach, Lamongan. All 17 isolates showed different macroscopic and microscopic characteristics. The most prevailing macroscopic characteristics of the 17 isolates were cream colored colonies with irregular shape and dull texture. TL-3 isolate showed most distinct macroscopic characteristics, as the colonies were shiny yellow. The diversity of bacterial isolates can be influenced by various factors such as soil pH and temperature. According to Msimbira and Smith (2020), most of the microbes can grow optimally in the pH range of 5.5-6.5. This pH range is also optimal for plant growth due to the optimal availability of nutrients, in this range plants can grow well and produce more root exudate as a source of available carbon for survival and microbial multiplication. This statement is supported by Walalangi (2020) that optimum growth temperatures in mangrove areas range between 28-32°C. The mangrove area in Labuhan Village, Lamongan has an average soil pH of 8.08 and an average temperature of 29.74°C. Haldar and Nazareth (2018) conducted a study related to bacterial diversity in mangrove forest sediments of Goa, India, with a soil pH of 6.5 and a mangrove area temperature of 35°C. 12 bacterial phyla were found with four isolates of which were phosphate solubilizing bacteria, namely *Bacillus toyonensis* (M022), and *Lysinibacillus macroides* (M002, M003, M005, Z010).

The nutrient content in the soil plays a role in biomass and bacterial diversity. According to Bastida et al. (2021), as the C content in the soil increases, the abundance of dominant bacterial taxa increases resulting in the increasing competition level so that the abundance of subordinate bacterial taxa decreases and the diversity of bacterial species in the soil becomes less abundant.

Isolates obtained from the mangrove rhizosphere soil of Kutang Beach, Lamongan were dominated by Gram-positive bacteria, as many as 14 isolates. In the present study, 15 bacteria were rod-shaped and only two bacterial isolates were cocci. Of the 17 isolates, 12 had the ability to dissolve phosphate. Phosphate solubilization activity was indicated by the formation of a clear zone around the colonies on Pikovskaya Agar media. Pikovskaya Agar media contains an insoluble phosphate compound, namely tricalcium phosphate. PSB can produce organic acids to form chelates with calcium, so that phosphate can be utilized. This process causes the clear zone to be formed. Phosphate solubilizing microorganisms have several mechanisms in dissolving insoluble phosphate compounds, namely by producing organic acids such as acetic acid, formic acid, lactic acid, gluconic acid, glycolic acid, oxalic acid, succinic acid, malic acid, and citric acid; form chelating substances with metal cations such as Al, Ca, and Fe; producing inorganic acids such as sulfuric acid, carbonic acid, and nitric acid; H^+ excretion affected by nitrogen supply; produce exopolysaccharides that can bind to metals in the soil; secrete siderophore substances which have high affinity for iron chelates; produce enzymes such as phosphatase which can hydrolyze phosphate ester bonds, phytase which can hydrolyze phytic acid, and C-P lyase which can hydrolyze ester bonds in phosphoenolpyruvate and phosphonoacetate (Prabhu et al. 2019).

Phosphate solubilization index is a value to measure PSB's ability to dissolve phosphate. The higher the phosphate solubility index value, the better the PSB's ability to dissolve phosphate. Results showed that TL-8 isolate had the highest (2.82) phosphate solubilization index value. In previous study, Fatimah et al. (2021), had successfully isolated 19 isolates from mangrove in Tuban East Java, the most potential PSB had a phosphate solubiliation index value of 2.36. The difference in the value of the phosphate solubilization index of each isolate was closely related to the ability of each isolate to dissolve the bound phosphate. The size of the PSB colony diameter was not always linearly correlated with the high value of the phosphate solubilization index, as well as the large diameter of the clear zone which was not always linearly correlated with the high phosphate solubilization index. The TL-13 isolate which had the highest colony diameter of 11.56 mm and clear zone diameter of 25.86 mm, respectively but the phosphate solubilization index value was not higher than other isolates such as TL-6, TL-8, TL-11, TL-15.

The gene encoding 16S rRNA is a gene that contains a highly conserved nucleotide sequence, shared by almost all types of bacteria, and there are certain sites that are identical to certain genus or even species of bacteria. Therefore, molecular identification of bacteria by utilizing the gene encoding 16S rRNA allows a more precise identification of bacteria (Jenkins et al. 2012). The 16S rRNA gene amplification of the TL-8 showed that the sequence length was around 1500 base pairs . The Bioedit analysis showed the length of sequence was 1405 bp.

BLASTn analysis showed that isolate TL-8 had 99.57% similarity to *L. fusiformis* strain NBRC 15717T.102 with a query cover of 100%. Thus, the query sequence (16S rRNA sequence belonging to the isolate TL-8) covered the entire target sequence (16S rRNA sequence belonging to *L. fusiformis*) and had 99.57% similarity. The molecular identification results were supported by the results of macroscopic characterization of TL-8 isolate, which had cream, irregular, and flat colonies where *L. fusiformis* also showed similar colony morphology. The TL-8 isolate showed a rod cell shape and was classified as a Gram-positive bacterium, similar to *L. fusiformis*.

Khaskheli et al. (2020) successfully isolated *L. fusiformis* from rice roots, one of the most common species of rice root endophytic bacteria. *L. fusiformis* was isolated from the stems of mangrove plant *Rhizophora mucronata* (Prihanto et al. 2020), roots of the mangrove plant *Sonneratia alba* (Handayani et al. 2018), apples (Bulgari et al. 2012), cereal plants (Damodaran et al. 2012), and tomatoes (Rahmoune et al. 2016). Several strains of *L. fusiformis* have been isolated from plants and investigated for their activity as plant growth promoting bacteria and functioning as biological control agents Rahmoune et al. 2016; Damodaran et al. 2018). The genus *Lysinibacillus* as dissolve phosphate bacteria have been isolated from alluvial and latosol soil paddy fields in the West Java, Indonesia which can selected as candidate strains for producing microbiological fertilizer (Ikhwani et al. 2021). Conversion of insoluble to dissolved minerals by *Lysinibacillus* spp. facilitated by the production of organic acids, hydrolytic enzymes, and metal chelating compounds (Naureen et al. 2017).

It was concluded that PSB isolates from the Lamongan mangrove area were dominated by Gram-positive bacteria. The most common macroscopic characteristics of 17 PSB isolates were cream-colored colonies with irregular shapes and dull textures. The TL-8 isolate was the most potent PSB isolate with the highest phosphate solubility index value of 2.82, and it can be utilized as potential candidate for biofertilizer. Molecular identification using the gene encoding 16S rRNA showed that isolate TL-8 had 99.57% similarity to *L. fusiformis* strain NBRC 15717T.102 with accession code MN543804 and identified as *L. fusiformis* TL-8.

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