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Isolation and characterization of indigenous phosphate solubilizing bacteria from calcareous soil of dry land ecosystems in Timor Tengah Selatan, East Nusa Tenggara, Indonesia

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Abstract. *Nur MSM, Benggu YI, Tae ASJA, Ishaq LF, Soetedjo INP. 2023. Isolation and characterization of indigenous phosphate solubilizing bacteria from calcareous soil of dry land ecosystems in Timor Tengah Selatan, East Nusa Tenggara, Indonesia. Intl J Trop Drylands 7: 66-72.* Phosphate Solubilizing Bacteria (PSB) is a potential biofertilizer due to its ability to increase phosphorus (P) availability. That is important, especially in areas where P availability becomes a constraint for plant growth, such as calcareous soil in Timor Island, East Nusa Tenggara, Indonesia. The study was undertaken in three ecosystems, including *mamar*, farm, and coastal areas in Timor Tengah Selatan, with the objective was obtaining and characterizing PSBs from these ecosystems. Five soil samples from the rhizosphere of five plants were collected from each ecosystem for PSB occurrence and soil physicochemical properties. The results showed that PSB could be found in these three ecosystems observed, with the highest types of the isolate being in the coastal area ecosystem compared to *mamar* and farm ecosystems. PSBs were relatively low in the three ecosystems, possibly related to the soil properties. Despite the low population of PSBs obtained, 19 isolates of PSBs were found in this study. The study results provide initial information on the occurrence of PSBs in calcareous soil Timor. This study needs to be expanded to screen and identify the PSB isolates used as biofertilizers for calcareous soil in the region.

Keywords: Calcareous soil, phosphate solubilizing bacteria, phosphorus

INTRODUCTION

Phosphorus (P) is an essential macronutrient plants require for normal growth and development. P is required by plants in relatively large amounts after nitrogen and potassium, contributing around 0.2 to 0.8% of plant dry weight (Havlin et al. 1999). Despite the high amount of P needed by plants, the availability of P to plants for uptake and utilization is generally low, and it has become one of the most important factors limiting plant growth (Leytem and Mikkelsen 2005). The availability of P is strongly controlled by soil pH, with the maximum solubility and availability at pH 6.5. Iron and aluminum phosphate formation in acidic soil results in decreased P solubility. In alkaline and calcareous soil, calcium readily adsorbs P, forming low-soluble calcium phosphate (Brady and Weil 2002).

Low availability of P is a general problem occurring in semi-arid Timor Island in Indonesia, including Timor Tengah Selatan, where the soils are calcareous. In this soil type, the mineral calcium carbonate ($CaCO_3$) is present in the parent material, and lime is accumulated in the soil (Leytem and Mikkelsen 2005). Accordingly, decreased P availability in alkaline soil is driven by the reaction of P with calcium, and a high amount of lime, particularly in the calcareous soil, could exacerbate the P availability problem. Since a high amount of lime in calcareous soil could react with soil solution P, it could form a strong calcium phosphate bond at the surface of the lime, resulting in low available P (Hopkins and Ellsworth 2005).

Due to the low concentration and poor mobility of soil available P, inorganic phosphate fertilizer is the main solution to crop growth and yield in the Timor Tengah Selatan Region. However, phosphate fertilizer applied in calcareous soil is relatively inefficient as the majority of phosphorus applied would be rapidly converted into forms that are less soluble compounds (Oxmann and Schwendenmann 2015), leading to reduced solubility and availability to plants (Brady and Weil 2002). As a consequence, phosphate fertilizer should be applied in high amounts. On the other hand, a high amount of inorganic fertilizer could imbalance the availability of other essential nutrients, and it is not environmentally safe. Therefore, an alternative approach should be considered in managing P's low availability in the region's calcareous soil. Furthermore, using soil amendments, such as biochar-fortified compost, could decrease un-available phosphate and increase available P and the absorption of P calcareous soil in Timor, East Nusa Tenggara, Indonesia (Nur et al. 2014). Moreover, beneficial functional soil microorganisms such as mycorrhiza have also been reported to improve available P and plant growth and yield in Timor's calcareous soil (Ishaq et al. 2021).

Another potential beneficial soil microorganism that is also known could also improve the availability of soil P is phosphate Solubilizing Bacteria (PSB). PSBs are known to be abundant in the rhizosphere of various plants and can solubilize potential phosphorus from organic and inorganic sources. It not only could produce phosphatase enzymes but also could secrete various low molecular-weight organic acids such as citric acid, glutamate, succinic, tartrates, formic acetic propionic lactonic glyconic and fumaric acids (Pande et al. 2017; Yadav et al. 2017). These organic acids can form a chelate (stable complex) with P-binding cations such as Fe^{2+} and $Ca^{2+,}$ resulting in reduced reactivity of these cations, leading to increased solubility and availability of P (Pande et al. 2017). Many studies have reported the potential of PSB to improve plant growth and yield and reduce the use of inorganic fertilizer applications (Hussain et al. 2019; Fitriatin et al. 2021; Rawat et al. 2021). Due to its benefit in increasing phosphorus availability and plant growth, PSB has been suggested as an essential biofertilizer that could reduce chemical fertilizer applications (Dhuldhaj and Malik 2022).

Despite the benefits provided by PSB, up to now, very little is known about the presence of these bacteria in the calcareous soil of dry land ecosystems of Timor Island. Therefore, the study aimed to obtain and characterize indigenous PSBs from the rhizosphere of plants in *mamar*, farm, and coastal areas in Timor Island's dry land ecosystems, particularly in Timor Tengah Selatan.

MATERIALS AND METHODS

Soil samples collection

Soil samples were collected from some ecosystems in the Timor Tengah Selatan District, East Nusa Tenggara, Indonesia, including *mamar*, farm, and coastal areas. *Mamar* is local farming management typical to Timorese farmers, which is located in water catchment areas with various plants grown such as coconut, bananas, areca nut, coffee, and other natural tree species such as bamboo and *Leucaena leucocephala* (Lam.) de Wit. Farm in Timor Tengah Selatan is typical of a semi-arid agricultural farming system in Timor consisting of various crops and plants such as maize, nuts, cassava, *Moringa oleifera* Lam, and *L. leucocephala*. The coastal area site was located around 100 m from the beach with a few natural vegetation such as *Jatropha gossypiifolia* L., *Borassus sundaicus* Becc., *Albizia* sp., and *Ziziphus mauritiana* Lam. These three ecosystems were chosen due to their specific characteristics, including soil and microenvironment. Before soil sample collection, a survey was undertaken to select the area of these three ecosystems that could represent each. The selection of sampling sites in each ecosystem was based on the diversity of plants growing there.

Five soil samples were collected diagonally in each ecosystem at 0-20 cm depth. The soil, around 1 kg, was taken from the rhizosphere of the plants, put in a labeled plastic zip bag, and then kept in a cool container. The location of the sampling point and the vegetation in each were recorded. The soil samples collected from each ecosystem (five soil samples from each ecosystem) were divided into two portions: one portion was used to isolate PSBs, and the rest of the soil was bulk for soil analyses representing the soil properties of the ecosystem. The soil physicochemical properties analyzed consisted of organic-C, total N, C/N ratio, available P, pH, and water content.

Isolation of PSBs

PSB strains were isolated from each soil sample by serial dilution and spread plate method. One gram (1 g) of soil sample was dispersed in 9 mL sterile 0.85% NaCl solution and thoroughly shaken. Next, 1 mL of that solution was transferred to 9 mL of sterile 0.85% NaCl to form a 10^{-2} dilution. The same method was used to make 10^{-3}, 10^{-4}, and 10^{-5} dilutions. Then, 1 mL of each dilution was spread on Pikovskaya's agar medium (PVK) and incubated at 28°C for 7 days. The PVK medium contained (in g/L) 10 glucose, 0.5 yeast extract, 0.5 $(NH_4)_2\ SO_4$, 0.1 $MgSO_4.7H_2O$, 5 $Ca_3(PO_4)_2$, 0.2 KCl, 0.002 $MnSO_4.2H_2O$, 0.002 $FeSO_4.7H_2O$, and 15 agar (Sharon et al. 2016). Colonies showing halo zones were picked and purified by 5 times subculture method on Pikovskaya's (PVK) agar medium for observing colony morphology. The colony with a morphologically distinct appearance with halo zones were selected and purified by 3 times subculture method on PVK agar medium to identify their colony characteristics and microscopic observation (Santoso 2007).

The population of PSBs was expressed as Colony-Forming Units (CFU) and counted based on Somasegaran and Hoben (1994) using the following equation:

Population (cfu/g soil) = (number of colonies × dilution factor) / (Volume of the aliquot)

The halo zones formed by the PSBs were calculated based on Sharon et al. (2016) using the equation below:

Phosphate solubilizing index = (colony diameter + halo zones) / colony diameter

The halozone and colony diameter were measured on large, medium, and small colonies. The colony is categorized as Large (L) if the diameter of the colony is above 2,100 µm. While the colony is classified as medium (M) if the diameter of the colony is in the range of 1,000-2,100 µm, and it is categorized as small (S) if the diameter of the colony is less than 1,000 µm. Measurement of colony diameter and halo zone was undertaken using a Hirox microscope at 100x magnification.

RESULTS AND DISCUSSION

General conditions of the study site

The location of the study sites (*mamar*, farm, and coastal area) is described in Figure 1. The detailed condition of the ecosystems where the soil was taken for isolation and characterization of the PSB in Timor Tengah Selatan District is shown in Table 1. *Mamar* ecosystem is located on Kobelete II, Cendana Village, So'e City Sub-district, with an altitude of 744-753 meters above sea level (masl). The vegetation in this ecosystem mainly consists of betel nut, bamboo, coconut, and some *L. leucocephala*. The farm ecosystem is located in Besipae, Pubabu Village, South Amanuban Sub-district, with an altitude of 174-177 masl. The plants grown in the farm ecosystem are *Carica* *papaya* L., pigeon pea, cassava, maize, and *M. olivera*. The coastal area ecosystem is located in Tuafanu Village, Kualin Sub-district, the altitude is 0 masl, and the natural vegetation consists of *J. gossypiifolia*, *B. sundaicus*, *Albizia* sp., and *Z. mauritiana*.

Soil analysis

The soil C-organic content in the study sites varied from moderate to very high. The highest C-organic content was found in the *mamar* ecosystem, accounting for 5.6%, followed by farm at 4.8%, and coastal area at 2.2%. Total nitrogen in the soil is low in all ecosystems (0.12-0.17%). The C/N ratio varied from high to very high. The highest C/N ratio was found in the *mamar* ecosystem (32.74), followed by the farm ecosystem (28.25) and the coastal ecosystem (22.20). Available P is very low in all ecosystems, ranging from 1.38 mg/kg to 2.47 mg/kg. The soil pH of *mamar* and the farm is neutral, while in the coastal ecosystem, the soil pH is slightly alkaline (7.80) (Table 2).

Table 1. Detailed condition of *mamar*, farm, and coastal area ecosystems where the soil samples were taken

Soil Sample	Type of Ecosystem								
	***Mamar* (Kobelete II)**			**Farm (Besipae)**			**Coastal Area (Tuafanu)**		
	Rhizosphere	**Altitude (m asl.)**	**Position**	**Rhizosphere**	**Altitude (m asl.)**	**Position**	**Rhizosphere**	**Altitude (m asl.)**	**Position**
1	Betel nut	745	9°52'26.634"S, 124°15'54.954"E	*C. papaya*	174	10°1'47.16"S, 124°12'9.396"E	*J.gossypifilia*	0	10°10'2.058"S, 124°23'46.794"E
2	Bamboo	744	9°52'26.262"S, 124°15'53.79"E	Pigeon pea	177	10°1'47.364"S, 124°12'9.834"E	*B. sundaicus*	0	10°10'2.562"S, 124°23'47.436"E
3	Coconut	753	9°52'26.688"S, 124°15'53.334"E	Cassava	176	10°1'48.09"S, 124°12'9.57"E	*Albizia* sp.	0	10°10'0.684"S, 124°23'47.082"E
4	*L.leucocephala*	750	9°52'27.87"S, 124°15'54.006"E	maize	175	10°1'47.886"S, 124°12'10.332"E	*Z.mauritiana*	0	10°10'1.578"S, 124°23'48.474"E
5	Banana Tree	751	9°52'27.144"S, 124°15'55.152"E	*M. olivera*	174	10°1'47.658"S, 124°12'10.098"E	*Borassus*	0	10°10'1.692"S, 124°23'47.736"E

Table 2. Soil physico-chemical characteristic of *mamar*, farm, and coastal area ecosystems

Soil physico-chemical characteristics	*Mamar* (Kobelete II)	Farm (Besipae)	Coastal Area (Tuafanu)
Organic-C (%)	5.57 (Very High*)	4.8 (High)	2.66 (Moderate)
Total-N (%)	0.17 (Low)	0.17 (Low)	0.12 (Low)
C/N Ratio	32.74 (Very High)	28.25 (Very High)	22.20 (High)
Available-P (mg/kg)	2.47 (Very Low)	1.38 (Very Low)	1.86 (Very Low)
pH	7.25 (Neutral)	7.56 (Neutral)	7.80 (slightly alkaline)
Water Content (%)	22.88	10.63	19.07

Note: *The criteria based on Hardjowigeno (2005)

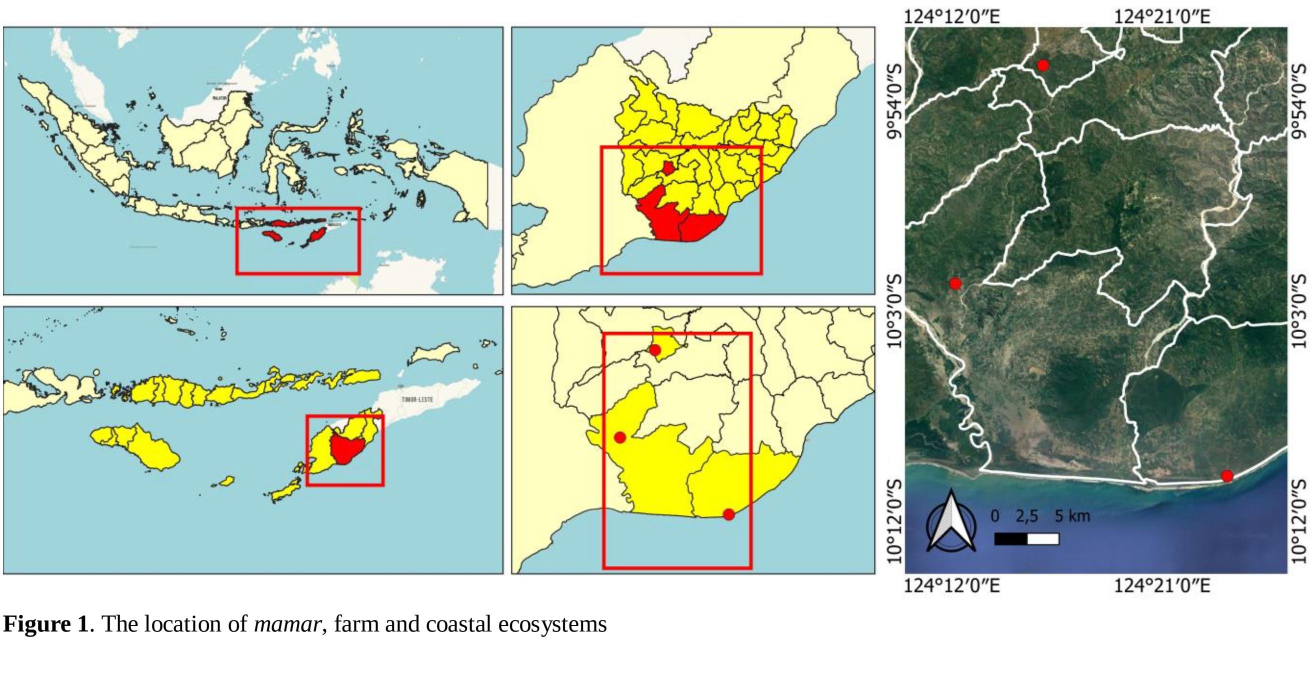

Figure 1. The location of *mamar*, farm and coastal ecosystems

Number of colonies, halo zones and colony diameter, and Phosphate Solubilizing Index (PSI) of PSBs in *mamar*, farm, and coastal area ecosystems

The population of PSBs observed in this study was relatively low as the colony was found in the first or second dilution. Most PSB was found in the coastal ecosystem, followed by the farm and *mamar* ecosystems. Particularly, in the *mamar* ecosystem, PSB was only found in the rhizosphere of bamboo and *L. leucephala*, while in the rhizosphere of betel nut, coconut, and banana samples, no PBS was observed. In the coastal area ecosystem, the highest population of PSBs was in the rhizosphere of *Z. mauritiana*, while in the farm ecosystem, the most populous PSBs were in the rhizosphere of pigeon peas (Table 3).

Although the halo zone diameter of the PSBs was quite variable between the three ecosystems, they were all small to medium categories. The diameter of the PSBs colonies ranges from 0.3 mm to 2.1 mm.

Macroscopic and microscopic morphological characteristics of PSB

The morphological appearances of PSB colonies are described in Table 4. The macroscopic characteristics of PSBs included in this study were shape, color, surface, margin, elevation, and size. The microscopic characteristics of PSB were the shape of the cell and gram staining. The shapes of colonies of PSB were circular and irregular. The color of the colony was observed as white, milky white, yellow, and green. The surface of the colony was shiny, smooth, and rough. The margin of the colony was entire, undulate, lobate, and serrate. The elevation of the colony was raised and flat. The size of the colony was pinpoint, small, and moderate. The cell shapes were monococcus, diplococcus, streptococcus, monobacil, diplobacil, streptobacil, and cocobacil, and the gram staining was positive and negative.

The types of colonies of PSB observed in the three ecosystems were different. There were 2 colony types found in the Mamar ecosystem, while in the farm and coastal area ecosystems, there were 8 and 9 colony types, respectively. The examples of the type of colony observed in three ecosystems are described in Figure 2.

Table 3. Number of colonies, the diameter of colony and halo zones, and Phosphate Solubilizing Index (PSI) of PSBs

Ecosystems	Rhizosphere origin colonies	Number of bacteria (CFU/g soil)	Phosphate solubilizing bacteria (PSB)		
			Mean of colony diameter(mm)	Mean of halo zones diameter (mm)	PSI
Mamar (Kobelete II)	Betel nut	0			
	Bamboo	33×10^{-1}	1.4	1.7	2.2
	Coconut	0			
	L. leucephala	46×10^{-1}	1.6	2.5	2.6
	Banana	0			
Farm (Besi pae)	*C. papaya*	68×10^{-2}	0.3	0.4	2.3
	Pigeon pea	93×10^{-1}	0.7	1.9	3.7
	Cassava	38×10^{-1}	2.1	2.7	2.3
	Maize	68×10^{-1}	0.7	1.1	2.6
	M. olivera	31×10^{-1}	1.2	2.0	2.7
Coastal area (Tuafa nu)	*J. gossypiifolia*	87×10^{-1}	0.8	1.4	2.7
	B. sundaicus	32×10^{-1}	1.6	2.7	2.7
	Albizia sp.	76×10^{-2}	0.5	0.6	2.2
	Z. mauritiana	114×10^{-1}	0.7	1.1	2.6
	B. sundaicus	55×10^{-1}	0.7	0.3	1.4

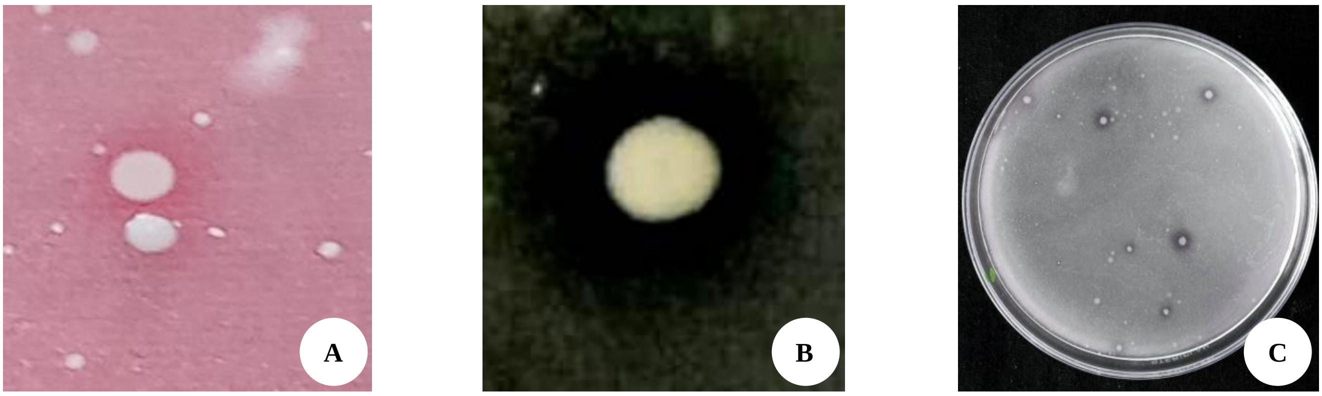

Figure 2. Examples of PSB colonies found in A. *mamar*, B. Farm, and C. Coastal areas ecosystems

Table 4. Macroscopic and microscopic characteristics of PSB isolated from *mamar*, farm, and coastal area ecosystems

Ecosystems	Isolate Origin	Macroscopic Characteristics of Colonies						Microscopic Characteristics of Colonies	
		Shape	Color	Surface	Margin	Elevation	Size	Shape of Cell	Gram Staining
Mamar	Betel nut tree	-	-	-	-	-	-	-	-
(Kobelete II)	Bamboo tree	Circular	Mw*	Ss	Entire	Raised	Small	Diplococcus	Negative
	Coconut tree	-	-	-	-	-	-	-	-
	L. leucephala	Circular	Mw	Ss	Entire	Raised	Small	Diplobacil	Negative
	Banana tree	-	-	-	-	-	-	-	-
Farm	*C. papaya*	Circular	White	Ss	Entire	Raised	Moderate	Cocobacil	Positive
(Besipae)	Pigeon pea	Circular	White	Ss	Entire	Raised	Small	Diplococcus	Positive
	Cassava	Circular	White	Ss	Entire	Raised	Moderate	Diplococcus	Positive
	Maize	Circular	White	Ss	Entire	Raised	Small	Streptobacil	Positive
	M. olivera	Circular	White	Ss	Entire	Raised	Small	Monobacil	Positive
	C. papaya	Irregular	White	Ss	Undulate	Flat	Small	Monococcus	Positive
	Pigeon pea	Circular	White	Ss	Entire	Flat	Moderate	Cocobacil	Positive
	Cassava	Circular	White	Ss	Entire	Flat	Small	Diplobacil	Positive
Coastal area	*J. gossypiifolia*	Circular	White	Ss	Entire	Raised	Small	Diplococcus	Negative
(Tuafanu)	*B. sundaicus*	Irregular	White	Ss	Lobate	Flat	Moderate	Diplococcus	Negative
	Albizia sp.	Circular	White	Rough	Entire	Raised	Small	Diplococcus	Negative
	Z. mauritiana	Circular	White	Ss	Entire	Raised	Pinpoint	Streptobacil	Negative
	B. sundaicus	Circular	Yellow	Ss	Entire	Raised	Small	Streptobacil	Negative
	J. gossypiifolia	Circular	Green	Rough	Serrate	Raised	Small	Diplobacil	Negative
	B. sundaicus	Circular	White	Ss	Entire	Raised	Small	Diplobacil	Negative
	Albizia sp.	Circular	Green	Rough	Entire	Raised	Small	streptococcus	Negative
	Z. mauritiana	Circular	White	Ss	Entire	Raised	Pinpoint	streptococcus	Negative

Note: *Mw: milky white, Ss: shiny smooth

Discussions

In this study, PSBs could be found in *mamar*, farm, and coastal area ecosystems. However, there is variability in the population and diversity of isolates obtained. The PSBs could be found in the five rhizospheres of plants in the farm and coastal area ecosystems, but in the *mamar* ecosystems, PSBs were only obtained in two rhizospheres of plants. Moreover, based on macroscopic and microscopic characterization, the number of isolates obtained was also low in the *mamar* ecosystems, with 2 isolates observed, compared with farm and coastal area ecosystems, with 8 and 9 isolates obtained, respectively. Many factors could affect the population and diversity of soil microorganisms. Plant species release various amounts and types of exudates, including carbohydrate, carboxylic acids, and amino acids, all of which can strongly affect the type of microorganisms colonizing the rhizosphere (Grayston et al. 1998).

In addition to host factors, soil properties such as organic carbon content, nitrogen, temperature, and aeration might influence the population and diversity of microorganisms, including PSBs (Mussarat and Khan 2014). In this study, the organic C content in the three ecosystems varies from moderate in the coastal area to high in the farm and very high in the *mamar* ecosystem. The lower organic C content in the coastal area ecosystem compared to the farm and *mamar* ecosystems is likely due to the lower population of vegetation in this area. Only a few vegetation in the coastal area ecosystem include *J. gossypiifolia*, *B. sundaicus*, *Albizia* sp., and *Z. mauritiana.* While in the farm and *mamar* ecosystems, the vegetation is more dense and variable, contributing more organic C resources. According to Chen et al. (2022), organic matter affects the properties of the soil, such as soil structure, moisture holding capacity, diversity and activity of soil organisms, and nutrient availability. However, this study found that the higher C organic content in the *mamar* ecosystem was unrelated to the population and diversity of PSBs. The higher population of PSBs was observed in the coastal area where the C organic content was lower than in *mamar* and farm ecosystems, which might be due to the C/N ratio of the soil. The organic matter with a lower C/N ratio is mineralized faster than those with a higher ratio (Havlin et al. 1999; Akratos et al. 2017), resulting in accelerated nutrient availability for supporting microorganisms and plant growth. In this study, the C/N ratio of the coastal area is lower than that in the *mamar* and farm ecosystems, indicating that the decomposition rate and nutrient supply in this area are faster than in the other two ecosystems.

Soil analysis showed that total-N in the three ecosystems is low. The low N soil content could explain the low population of PSB in the three ecosystems observed. Nitrogen is a crucial nutrient factor that could influence soil-inhabiting microbes' growth and functionality, including PSBs. If soil N content is low, it affects the growth and development of all microorganisms (Sandle 2016), including PSB (Mussarat and Khan 2014). In this study, all samples collected from each location were bulk to represent the soil-N content in that location. Therefore, it is hard to draw a correlation impact between soil total-N and its effects on the PSB population due to a low number of soil samples. In the future, more soil samples must be collected to evaluate the relationship between soil N and its effect on the PSB population.

In addition to N, the availability of P is also very low in the three ecosystems. The low availability of P in the ecosystems of Timor Island, including the Timor Tengah Selatan Region, is not surprising as the soil in the region is calcareous. Calcareous soils have high Ca content, which can adsorb P into insoluble Ca-P compounds (Brady and Weil 2002; Leytem and Mikkelsen 2005; Zhang et al. 2014). It is unknown whether the low availability of P in the soil could be related to the low population of PSBs in the study area; this needs to be further studied.

According to Mussarat and Khan (2014), aeration is one of the important factors that could influence PSB functionality in solubilizing phosphate. It has been reported that increasing the aeration rate to 0.4 L/kg DM min^{-1} was more conducive to enhancing P solubilization efficacy and available P accumulation than 0.2 L/kg DM min^{-1} (Ma et al. 2022). Related to the soil condition of the three ecosystems observed, it is likely that soil aeration in the *mamar* ecosystem could be less than in the farm and coastal ecosystems due to denser vegetation and higher organic matter content. This soil's physical conditions need to be investigated to evaluate this relationship.

The results showed that the phosphate solubilizing index was similar among the isolates obtained from *mamar*, farm, and coastal area ecosystems, with a value range of 2.2 to 2.7. The PSI of the isolates obtained in this study was slightly higher than the PSI of PSB isolates found in the mangrove rhizosphere in East Java, Indonesia (Fatima et al. 2023). That may indicate that the isolates found could be potential for biofertilizer purposes in the calcareous soil in the region. Therefore, further study was needed to screen and select the most potential isolate for biofertilizer use. In addition, since the characterization of the PSBs in this study was merely based on the macroscopic and microscopic characteristics of PSBs, there is a need to identify the potential isolated PSBs in the future using molecular analysis.

In conclusion, the results showed that the population of PSB isolates obtained in this study is variable between the ecosystems, and it is relatively low, possibly related to the soil's chemical characteristics. The population and types of PSB isolates are higher in the coastal area than in the *mamar* and farm ecosystems. There are 19 types in total of PSBs found in this study with different macroscopic and microscopic appearances.

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