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Diversity and phosphate solubilization by bacteria isolated from Laki Island coastal ecosystem

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ABSTRACT

Widawati S (2011) Diversity and phosphate solubilization by bacteria isolated from Laki Island coastal ecosystem. Biodiversitas 12: 17-21. Soil, water, sand, and plant rhizosphere samples collected from coastal ecosystem of Laki Island, Jakarta, Indonesia were screened for phosphate solubilizing bacteria (PSB). While the population was dependent on the cultivation media and the sample type, the highest bacterial population was observed in the rhizosphere of *Ipomoea aquatica*. The PSB strains isolated from the sample registered 18.59 $g^{-1}L^{-1}$, 18.31 $g^{-1}L^{-1}$, and 5.68 $g^{-1}L^{-1}$ of calcium phosphate (Ca-P), Al-P and rock phosphate solubilization after 7-days. Phosphate solubilizing capacity was the highest in the Ca-P medium. Two strains, 13 and 14, registered highest phosphomonoesterase activities (2.01 µgNP.$g^{-1}.h^{-1}$ and 1.85NP µg.$g^{-1}.h^{-1}$) were identified as *Serratia marcescens*, and *Pseudomonas fluorescens*, respectively. Both strains were isolated from the crops of *Amaranthus hybridus* and *I. aquatica*, respectively, which are commonly observed in coastal ecosystems. The presence of phosphate solubilizing microorganisms and their ability to solubilize various types of phosphate species are indicative of the important role of both species of bacteria in the biogeochemical cycle of phosphorus and the plant growth in coastal ecosystems.

Key words: phosphate solubilization, coastal ecosystem, *Serratia marcescens, Pseudomonas fluorescens*.

INTRODUCTION

Soil microorganisms play a significant role in the food chain and the various biogeochemical cycling of carbon, nitrogen, sulfur and phosphorus (Kummerer 2004; Das et al. 2007; Banig et al. 2008). Though phosphate solubilizing bacteria (PSB) like *Pseudomonas, Serratia, Bacillus, Flavobacterium* and *Corynebacterium* have been reported from various ecosystem types such as terrestrial ecosystem especially soil, and water ecosystem including coastal, offshore and mangrove ecosystems (Seshadri et al. 2002; Young et al. 2006), their population has been occasionally very low, about less than 10^{-2} CFU per gram (Peix et al. 2004). A series of observation indicates that the highest population of PSB (an average of 10^8 CFU per gram of sample) was found in fertile soil of forests, organic farming and rhizosphere (Widawati et al. 2005; Widawati and Suliasih 2006; Widawati and Suliasih 2009), as well as mangrove and shorelines (De Souza et al. 2000).

Phosphate solubilizing bacteria form sea sediments were reported to be capable of accelerating the dissolution of apatite phosphate within the phosphorus cycle interacting with the carbon cycle (Thingstad and Rossouzadegan 1995). Phosphate solubilizing bacteria (PSB) living in both terrestrial and water ecosystem play a vital role in supplying phosphorus to plants (Gyaneshwar et al. 2002; Widawati and Rahmansyah 2009), which improves and maintain the fertility of farmlands (Shekar et al. 2000). They are capable of producing organic acids (acetate, formate, lactate, oxalate, malate and citrate), amino acids, vitamin and growth promoting substances like (IAA/indole-3-acetic acid) as well as gibberellic acid that stimulate the growth of the plants (Richardson 2001; Gyaneshwar et al. 2002; Ponmurugan and Gopi 2006). The mineralization of organic phosphate have been reported to be mediated through the production of phosphatase enzymes (phosphomonoesterase, phosphodiesterase, triphosphomonoesterase and phosphoramidase) which hydrolyse organic-P into $H_2PO_4^-$ and $HPO_4^=$ (Pang and Kolenk 1986; Mearyard 1999; Lal 2002).

Research on the phosphatase enzyme can benefit sustainable organic farming systems especially in coastal ecosystems, and reduce the utilization of agrochemicals in agricultural fields in Indonesian coasts. As isolation of PSB from the highly saline farmlands have been termed important prior to the use of PSB as biofertilizer in those areas (Bashan et al. 2000), this study was conducted to determine the potentials of PSB isolated from Laki Island coasts, Jakarta bay, Indonesia and surroundings to enhance the organic farming in the high saline areas.

MATERIAL AND METHODS

Isolation and enumeration of PSB

Samples were collected from soil and rhizosphere of plant in coastal areas, mangrove, and sea water (Figure 1). About 0.2 mL of serially diluted sample was put into a sterile petridish containing Pikovskaya's medium - PM1 [5

g of $Ca_3(PO_4)_2$. 0.5 g of $(NH_4)_2SO_4$; 0.2 g of NaCl; 0.1 g of $MgSO_4$. $7H_2O$; 0.2 g of KCl; 10 g of Glucose; 0.5 g of yeast extract; 20 g of agar; $MnSO_4$ and a little $FeSO_4$, 1000 l^{-1} of aquadest] (Gaur 1981). For the preparation of other media, the same media composition was retained but were changed as follows; PM1 + 1% NaCl (media 2), PM1 substituted with seawater (media 3), PM1 with $Al_3(PO_4)$ (media 4), PM1 with rock phosphate (media 5). All the flasks were incubated at 30°C and observed for 3 to 7 days for bacterial growth. Population was enumerated daily by plate count method (Rao 1982). The PSB showing formation of halozones on around the colonies were scored as positive. The colony of PSB was then purified and sub-cultured in Pikovskaya's medium for further studies.

PSB qualitative test

The size of halozones determines the ability level of microbes to dissolve the bonded P The purified PSB were then streaked on plates to test their ability to dissolve $Ca_3(PO_4)_2$, $Al_3(PO_4)$ and rock phosphate. The solubilization was marked with halozone formation around the growing colonies and the halozones size were expressed as solubilization efficiency as described by Nguyen et al. (1992) and Seshadri et al. (2002) method: E = Solubilization diameter/growth diameter x 100.

Broth assay

The PSBs were grown in 100 mL Pikovskaya broth in 250 mL Erlenmeyer flasks containing 5 g L^{-1} of $Ca_3(PO_4)_2$ and $Al_3(PO_4)$ (Gaur 1981). Each flask was inoculated with 1 mL (10^9 cfu mL^{-1}) of culture and incubated in a rotary shaker at 120 rpm and 30°C for 7 days. The cultures were centrifuged and analyzed for soluble phosphates (Allen, 1974), pH, and PME activity.

pH, acid and alkaline of PME

The pH of culture supernatant was measured directly using a pH meter. The phosphomonoesterase activities (acid and alkaline) were measured following the technique in Schinner et al. (1996). One mL of culture supernatant was added to 1 mL of 115 mM p-NPP phosphate substrate and 4 mL of buffer at pH 6.5 for acid PMEase and pH 7.5 for alkaline PMEase and incubated at 38°C for 1 hour. Subsequently 1 mL 0.5M $CaCl_2$ was added to the reaction. A control reaction was also maintained along with the sample. The absorbance of both the sample and control was measured spectrophotometrically at 400 nm.

Identification of PSB

Identification of the best isolates was carried out through a 16S RNA analysis (Pitcher et al. 1989). The method of amplification and sequencing of 16S rDNA according to Rivas et al. (2001) and the sequence obtained was compared with those in GenBank by using the FASTA program (Pearson and Lipman 1988).

RESULTS AND DISCUSSION

Population of PSBs observed from different environmental samples grown on various media is shown in Table 1. Only Fifteen isolates from twenty five isolates showed formation of halozones around the growing colonies. Phosphate tested ($Ca_3(PO_4)_2$. $Al_3(PO_4)$ and rock phosphate) were clearly dissolved by growing colonies (Table 1). Similar results was also found by Antoun (2009, pers. comm.), which proves that only 10 out of 31 tested bacteria were able to form halozones within the media that contain Ca-P and Al-P as the source of P. Halozones are formed because of transformation of glucose into organic acids (Sudiana 2010, personal communication). Glucose was the carbon source used in the test of PSB isolated from the sea to dissolve the bonded phosphor marked by halozones (De Souza et al. 2000). De Souza et al. (2000) and Seshadri et al. (2002) reported that the PSB isolated from coastal, offshore, mangrove and sea water were able to dissolve P from zinc phosphate (30%), from calcium triphosphate (19%) and from calcium triphosphate (18%).

Table 1. Population of PSB in various types of habitats enumerated using five different media; and phosphate dissolution by PSB on plates containing five different media

Altitude asl. (feet)	The ecosystem type of sample collected		Isolate no.	Population of PSB (∑pop.10^7)					Phosphate dissolution by PSB (EP)				
				1	2	3	4	5	1	2	3	4	5
0	Sediment, mangrove, Banten	(1)	SMJ	12 cd	11 ab	9 ab	0.9 a	7 ab	200 a	100 a	100 a	80 a	50 a
0	Sediment, mangrove, Laki Island	(2)	SMLI	12 cd	11 ab	9 ab	0.8 a	7 ab	200 a	100 a	100 a	90 a	50 a
0	Sand, Coastal area, Jakarta	(3)	SCJ	11 cd	10 ab	9 ab	0.8 a	7 ab	200 a	150 a	100 a	100 a	50 a
0	Sand, Coastal area, Laki Island	(4)	SCLI	11 cd	10 ab	8 ab	0.8 a	6 b	200 a	150 a	100 a	90 a	50 a
0	Sea water, 50 meter from Jakarta	(5)	SWJ50	8 d	9 b	7 ab	0.7 ab	5 b	200 a	200 a	100 a	80 a	50 a
0	Sea water, 50 meter from Laki Island	(6)	SWLI50	8 d	9 b	6 b	0.6 ab	5 b	200 a	100 a	100 a	100 a	50 a
0	Sea water (between Jakarta-Laki Island)	(7)	SWJLI1	7 d	9 b	9 ab	0.5 b	4 b	200 a	150 a	100 a	100 a	100 a
0	Sea water (between Jakarta-Laki Island)	(8)	SWJLI2	7 d	9 b	8 ab	0.4 b	8 ab	200 a	150 a	100 a	70 a	100 a
23	Rhizosphere mangrove, Banten	(9)	RMJ	15 c	12 ab	7 ab	0.9 a	9 ab	200 a	100 a	100 a	80 a	100 a
32	Rhizosphere mangrove, Laki Island	(10)	RMLI	15 c	12 ab	8 ab	0.8 a	10 ab	200 a	200 a	100 a	100 a	150 a
32	Rhizosphere *L. leucocephala*, Jakarta	(11)	RLJ	27 b	13 ab	7 ab	0.9 a	10 ab	200 a	200 a	100 a	100 a	100 a
32	Rhizosphere *O. sativa*, Banten	(12)	ROJ	30 b	13 ab	9 ab	0.6 ab	10 a	200 a	200 a	100 a	100 a	100 a
32	Rhizosphere *A. hybridus*, Banten	(13)	RAJ	32 b	14 a	10 a	0.9 a	11 a	200 a	200 a	200 a	200 a	200 a
32	Rhizosphere *I. aquatica*, Banten	(14)	RIJ	40 a	14 a	10 a	0.9 a	11 a	200 a	200 a	200 a	200 a	200 a
32	Soil without plant	(15)	SWP	15 c	10 ab	7 ab	0.6 ab	10 ab	200 a	200 a	100 a	90 a	100 a

Note: Alphabets followed by the same letters in the column are not significantly different analyzed through Duncan's Multiple Range Test (0.5% level). Nos. 9-15 are the ebb and tide farmlands in the coastal areas. Ability of phosphate dissolution by PSB were recorded after 7-days.

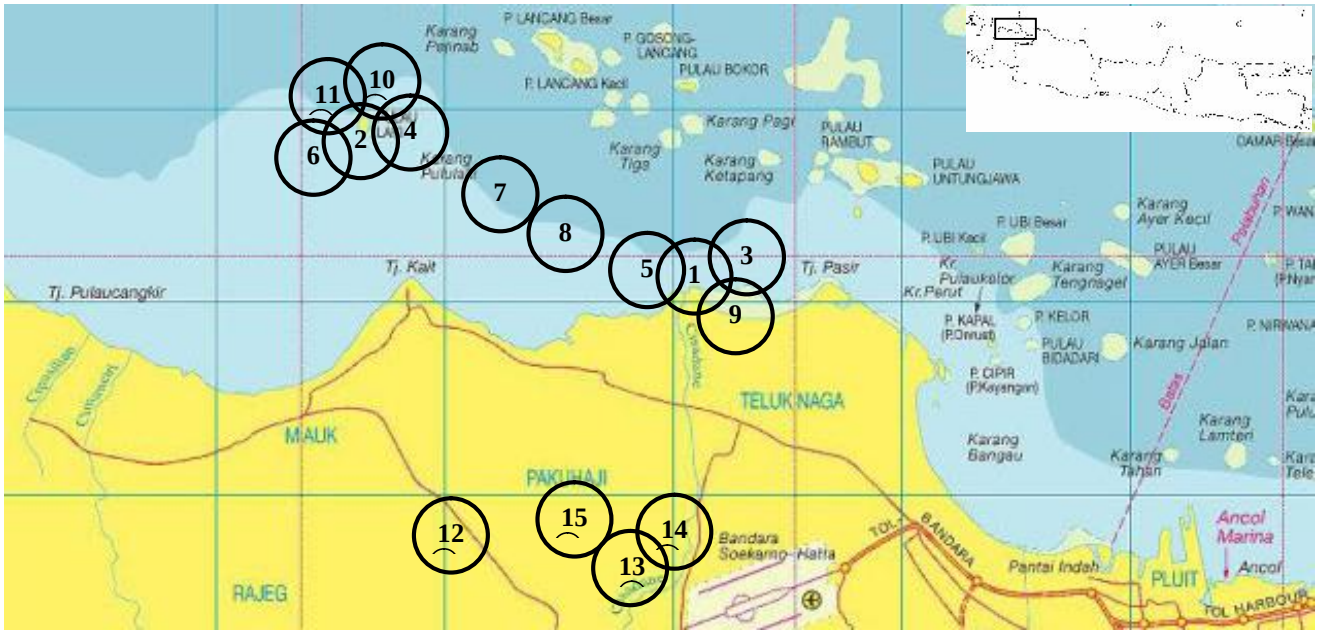
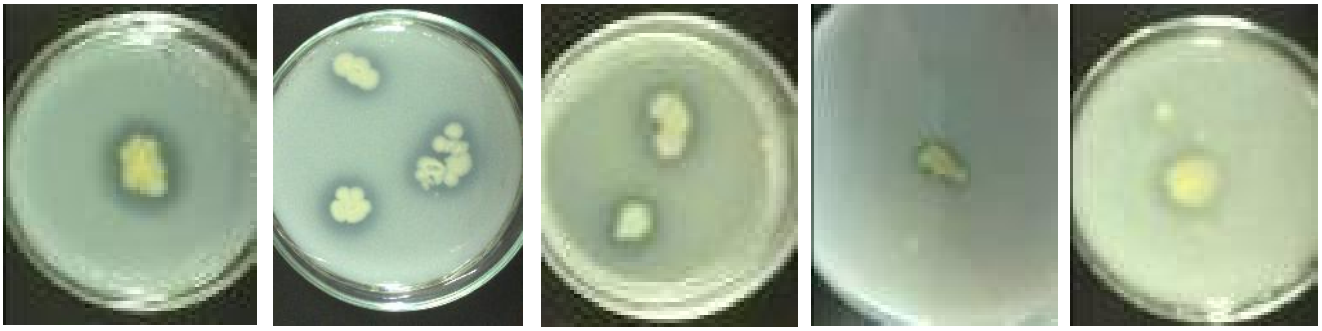


Figure 1. Sampling site location. No. of 1-15 refer to Table 1.



PM 1 **PM 2** **PM 3** **PM 4** **PM 5**

Figure 2. The ability of isolate 14 to dissolve the bonded phosphor within 5 types of Pikovskaya media (PM) and the isolate was identified as *Pseudomonas fluorescens* species.

Though maximum P solubilization efficiency of 15 was observed in two isolates 13 and 14 isolated from rhizosphere of *Amaranthus hybridus* and *Ipomoea aquatica*, there were differences among the fifteen isolates (Table 1). The size of halozones determines the ability level of microbes to dissolve the bonded P (Rachmiati 1995). The more the size of the halozones formed, the more likely the isolates dissolving the bonded P (Figure 2).

The PSB population from 15 in 5 types of pikovskaya media show that highest population observed in rhizosphere sample of *I. aquatica* plant. The average number was $40x10^7$ (media 1), $14x10^7$ (media 2), $10x10^7$ (media 3), $0.9x10^7$ (media 4), and $11x10^7$ (media 5). Thus, more population of PSB was observed in terrestrial (rhizosphere soil) than in water (sea, coastal areas, offshore areas and mangrove). The results can be seen in Table 1 and were supported by a research conducted by Kucey et al. (1989), which shows that the population of PSB in the rhizosphere areas is more than that in the areas without vegetation. Research conducted by De Souza et al. (2000) and Seshadri et al. (2002) reveals that the population of PSB on backwaters (16.7%) is more than that in the coastal areas (15.6%), in the offshore areas (8.7%), and in the beaches (8.1%). This is due to the fact that the organic phosphate is rare in the offshore areas and thus the population of PSB is low. The growth of PSB is impeded with the absence of soluble organic P (Hoppe and Ullrich 1999) and low absorption of C (De Souza et al. 2000).

The ability of PSB to quantitatively dissolve phosphate and pH of the liquid media with various P sources after 7-day incubation was measured using spectrophotometer. It shows that all of the tested isolates were able to dissolve the inorganic phosphate of $Ca_3(PO_4)_2$, $Al_3(PO_4)_2$ and rock phosphate within the medium of liquid Pikovskaya. The highest concentration of dissolve P was found in isolate number 14: 18.59 L^{-1}, 18.31 L^{-1} and 5.68 L^{-1} (Table 2). Meanwhile, isolate number 13 dissolves the highest P source only in rock phosphate. The lowest concentration was found in the isolates from the sea water, namely isolates number 7 and 8 (Table 3). When the phosphate in 5 media of liquid pikovskaya was dissolved, the pH in all of the media decreased (Table 3). The dissolution of

Table 2. P dissolved by PSB, pH, PME-ase acid and base after 7-day incubation in pure culture using several source P, namely calcium phosphate ($Ca_3(PO_4)_2$), alumunium phosphate ($Al_3(PO_4)_2$), and rock phosphate

The ecosystem type of sample collected		Calcium phosphate				Alumunium phosphate				Rock phosphate			
		Solubilize $Ca_3(PO_4)_2$	pH	PME-ase asam (µgNP.g^{-1}.h^{-1})	PME-ase basa (µgNP.g^{-1}.h^{-1})	Solubilize $Al_3(PO_4)_2$	pH	PME-ase asam (µgNP.g^{-1}.h^{-1})	PME-ase basa (µgNP.g^{-1}.h^{-1})	Solubilize rock phosphate	pH	PME-ase asam (µgNP.g^{-1}.h^{-1})	PME-ase basa (µgNP.g^{-1}.h^{-1})
Sediment, mangrove, Jakarta	(1)	7.34 de	5.8 ab	0.15 c	0.26 b	5. 16 d	4.1 cd	0.22 bc	0. 19 cd	3.39 b	5.9 b	0.24 bc	0.11 c
Sediment mangrove, Laki Island	(2)	7.11 de	4.5 c	0.15 c	0.29 b	5. 16 d	4.4 cd	0.45 b	0.25 cd	2.88 b	4.7 c	0.21 bc	0.34 a
Sand, Coastal area, Jakarta	(3)	6.96 e	5.5 ab	0.36 c	0.12 bc	2. 89 e	5.8 ab	0.16 bc	0.17 cd	0.81 c	3.6 ab	0.20 bc	0.13 c
Sand, Coastal area, Laki Island	(4)	2.11 f	5.9 ab	0.12 cd	0.14 bc	2.62 ef	5.8 ab	0.21 bc	0.27 cd	0.63 c	5.9 b	0.03 c	0.06 dc
Sea water, 50 meter from Jakarta	(5)	2.66 f	5.8 ab	0.10 cd	0.12 bc	3.43 de	5.8 ab	0.25 bc	0.16 cd	0.45 c	5.9 b	0.05 c	0.02 d
Sea water, 50 meter from Laki Island	(6)	1.45 f	6.0 ab	0.13 cd	0.09 c	3.43 de	4.7 cd	0.30 bc	0.25 cd	0.25 c	7.0 ab	0.06 c	0.05 d
Sea water (between Jakarta-Laki Island)	(7)	0.22 g	6.8 a	0.02 d	0.01 d	0.63 f	6.7 a	0.08 c	0.04 e	0.05 d	7.3 a	0.02 c	0.03 d
Sea water (between Jakarta-Laki Island)	(8)	0.25 h	6.8 a	0.04 d	0.01 d	1.69 f	6.9 a	0.08 c	0.91 d	0.04 d	7.4 a	0.05 c	0.30 ab
Rhizosphere, mangrove, Jakarta	(9)	10.45 cd	4.4 cd	0.27 c	0.21 b	9.92 c	5.6 bc	0.36 b	0.54 c	2.02 c	5.7 bc	0.41 b	0.19 bc
Rhizosphere, mangrove, Laki Island	(10)	11.24 bc	3.6 de	0.27 c	0.37 b	10.96 c	4.0 d	0.44 b	0.34 cd	2.44 bc	5.5 bc	0.55 b	0.29 ab
Rhizosphere *I. leucocephala*, Jakarta	(11)	11.45 bc	3.9 de	0.65 bc	0.19 b	14.68 b	3.7 d	0.55 b	0.38 cb	3.45 b	5.7 bc	0.36 b	0.35 a
Rhizosphere *O. sativa*, Jakarta	(12)	14.25 b	3.0 e	1.60 ab	1.51 a	14.81 b	3.1 de	1. 62 **a**	1.79 ab	3.75 a	4.9 bc	2.74 a	0.47 a
Rhizosphere, *A. hybridus*, Jakarta	(13)	14.94 b	2.3 e	2.01 a	1.85 a	17.41 a	3.3 de	2.01 a	1.36 b	4.72 a	4.7 c	3.14 a	0.57 a
Rhizosphere *I. aquatica*, Jakarta	(14)	18.59 a	2.6 e	2.01 a	2.01 a	18.31 a	2.8 de	2.01 a	1.92 a	5.68 a	4.5 c	3.23 a	0.57 a
Soil without plant	(15)	7.17 de	4.2 cd	0.33 c	0.48 b	11.35 c	5.6 bc	0.40 b	0.35 cd	3.39 a	4.2 c	0.22 b	0.26 abc

Note: the numbers followed by the same letters in the same column show slight difference in the level of 0.5% of Duncan test.

phosphate involving alteration in pH with the occurrence of synthetic organic compounds released to the medium, oxidation-reduction reactions, and organic ligand competitor (Cunningham and Kuiack 1992). A test conducted by Jeon et al. (2003) shows that *Pseudomonas fluorescens* dissolves $Ca_3(PO_4)_2$ after 5-day incubation and pH drops to 4.4. Perez et al. (2007) stated the acidity of a liquid culture is the main mechanism of phosphate dissolution. Whitelaw et al. (1999) also stated that the concentration of phosphate dissolved in the culture media is in line with the acidity and concentration of amino acid as well as pH shift. Rao (1982) stated that the mechanism of inorganic phosphate dissolution, including Ca_3PO_4, involves pH alteration due to the production of organic acids, such as acetate, citrate and oxalate. Ramachandran et al. (2007) stated that since the isolate is able to release inorganic phosphate from $Ca_3(PO_4)_2$ in the liquid media, the bacteria have the potentials to dissolve the bonded P and thus make it available for the plants.

All of the isolates were grown in the media using 3 different sources of phosphate ($Ca_3(PO_4)_2$), $Al_3(PO_4)_2$, and rock phosphate). All of the tested isolates produce fosfomonoesterse enzyme (PME-ase acid and base). Isolate 13 reaches the highest value, namely: 2.01 ugPNP $g^{-1}h^{-1}$ (PME-ase acid), 1.85 ugPNP $g^{-1}h^{-1}$ (PME-ase base), 2.01 ugPNP $g^{-1}h^{-1}$ (PME-ase acid), 1.36 ugPNP $g^{-1}h^{-1}$ (PME-ase base), and 3.14 ugPNP $g^{-1}h^{-1}$ (PME-ase acid), 0.57 ugPNP $g^{-1}h^{-1}$ (PME-ase base) (Table 2). Isolate number 14 also reaches the highest value: 2.01 ugPNP $g^{-1}h^{-1}$ (PME-ase acid and base), 2.01 ugPNP $g^{-1}h^{-1}$ (PME-ase acid) and 1.92 (PME-ase base), and 3.23 ugPNP $g^{-1}h^{-1}$ (PME-ase acid) and 0.57 ugPNP $g^{-1}h^{-1}$ (PME-ase base) (Table 2).

The highest activities of both acid enzyme and base enzyme were found in the PSB isolated from the soil where *Amaranthus hybridus* and *Ipomoea aquatica* plants grow. The tests show that the activities of the enzymes were determined by the number of PSB population, habitat types and vegetation types. However, the research conducted by Pang and Kolenk (1986) reveals that the activities of the enzyme are determined by types of vegetation that grow on the surface and yet the PSB population has no impacts on the activities.

The activities of enzyme PMEase-acid and base increased during incubation. This may due to induced when the amount of P in the culture growth was limited, which proves that P is much in demand (Savine et al. 2000). The activity of PMEase-acid and base reach its lowest and highest point during the 7-day incubation (Table 2). Phosphate is released from fosfomonoester by phosphomonoesterase through an enzyme-based hydrolysis (Ponmurugan and Gopi 2006).

Research shows that isolate number 13 and 14 were the most excellent P-solubilizing capacity throughout all tests (Table 1, 2, 3). Those isolates were identified using gene 16S RNA analysis (Pitcher et al. 1989). It shows that isolate number 13 and 14 belongs to the species *Serratia marcescens* and *Pseudomonas fluorescens*, with homology reaching 99%. The results support and elaborate the previous research. Rodriguez and Fraga (1999), for example, stated that *Pseudomonas* was the strain that best

dissolves the bonded P from any source of P and is dominant in the mineralization of organic phosphorus. This type of bacteria is found as a dominant cluster primarily on East Indian coast (Seshadri et al. 2002) and in on extreme saline water habitat and alkaline (De Souza et al. 2000).

CONCLUSION

Phosphate solubilizing bacteria (PSB) were found in the coastal, offshore, shorelines and mangrove ecosystem indicating that their wide distribution. *Serratia marcescens*, and *Pseudomonas fluorescens* were isolated from the rhizosphere of *Amaranthus hybridus* and *Ipomoea aquatic* respectively showed highest population in media 1, 2, 3, 4 and 5, and dissolved wide range of P including Al-P, Ca-P and rock phosphate and also produced both alkaline and acid phosphatases.

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