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Characterization of Phosphate Solubilising Bacteria from Limestone Quarry in Cirebon Indonesia

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Abstract: Limestone quarry has low available P for plant on land revegetation process. Therefore, phosphate-solubilising bacteria could be a solution to dissolve phosphate in limestone in order to add phosphate availability. The research was aimed to characterize and identify the phosphate-solubilising bacteria adapted in limestone quarry. Isolation was performed using Pikovskaya medium which obtained four isolates bacteria had halo-zone around the colony. The ability to dissolve phosphate was examined using five soil samples from different region of limestone quarry. The result showed index of P solubility between 1.63 to 3.42. Identification bacterial isolates by using 16S rRNA gene showed that the isolates have similarity with *Pseudomonas mosselii* QC5A.1, *Pseudomonas mosselii* QC5B.1, Pantoea ananatis QC5C.1, and Pseudomonas oryzihabitans QC5B.3.

Keywords: *Limestone quarry, phosphate-solubilising bacteria, Pseudomonas, Pantoea, 16S rRNA.*

Introduction

Phosphate (P) is a main macronutrient compound which is essential for plant growth. Problem commonly encountered in mine reclamation or re-vegetation is that pioneer plants planted in that area unable to easily get nutrients, especially phosphate (P). Plant roots cannot dissolve P compound in calcareous rock. Therefore it is necessary role of soil microorganisms to dissolve P compound which allow it to be available in easily processed formed for plant roots. These microorganisms can be isolated from the area and expected to have adapted to limestone conditions. However, among bacteria strains capable to solubilise P such as *Burkholderia, Serratia,* and *Pseudomonas* have found from buffer zone and reclamation area of limestone quarry at Cirebon, Indonesia (Mursyida *et al*., 2015).On the field application not all phosphate solubilising bacteria able to thrive and help plant re-vegetation process, because of the difference of nutrients and compound bond in each quarry. For example, the existing metal-phosphate bond in acidic pH land in the form of Fe-P and Al-P, and phosphate will bond with calcium (Ca-P) in alkaline condition which is more stable but insoluble, so that plant will be unable to absorb phosphate (Bashan *et al.,* 2013). This research was conducted to characterize and identify phosphate dissolving bacteria in Cirebon limestone quarry area, and to measure the ability of the bacteria on dissolving of phosphate in limestone quarry soil.

Materials and Methods

Soil Sampling Location

Soil sampling was conducted in limestone quarry, located in Palimanan, Cirebon, West Java Indonesia (PT Indocement Tunggal Prakarsa, Tbk). Sample was taken from five location, in which two locations from limestone active quarry (S06°43'08.2", E108°23'08.38" and S06°43'19.5", E108°24'04.6"), one location from non-reclamation area (S06°35'24.11", E106°48'21.2"), and two locations from reclamation area (S06°43'09.5", E108°24'01.5" and S06°43'18.6", E108°24'06.2"). Soil samples were taken approximately 1 kg from each sampling site in each location.

Chemical Analysis of Soil from Limestone Area: The chemical contents of limestone soil was measured of phosphorus (P), potassium (K), calcium (Ca), aluminium (Al), iron (Fe), manganese (Mn), magnesium (Mg), sodium (Na), chloride (Cl), soil pH, and water content (Gholizadeh *et al.* 2009).

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Isolation of Phosphate Solubilising Bacteria

As much as 10 g of soil sample was suspended in 90 ml sterile water in a 250 ml Erlenmeyer and shaken for 30 minutes. A serial dilution was carried out up to 10^{-5} dilution and then spread on Pikovskaya agar consisted of 10 g glucose, 5 g $Ca_3(PO_4)_2$, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.5 g yeast extract, 0.2 g KCl, 0.2 g NaCl, 0.002 g $FeSO_4 \cdot 7H_2O$, 0.002 g $MnSO_4 \cdot 7H_2O$, and 15 g bacto agar (per litter), the pH adjusted 7.5. After two days incubation at room temperature ($\pm$ 27 $^{\circ}$C), the colony of phosphate solubilising bacteria was surrounded by clear zones (halo-zone) (Nautiyal, 1999). Phosphate Solubility Index (SI) was calculated by using the following equation:

$$SI = \frac{\text{halo-zone diameter (cm)} - \text{colony diameter (cm)}}{\text{colony diameter (cm)}}$$

Morphology and Biochemical Identifications

Morphological characteristic of phosphate solubilising bacteria was observed by using Gram staining. Biochemical characteristic of the bacteria was known by using API kit (*Biomerieux*^{TM}, USA) and verified based on Bergey's Manual of Determinative Bacteriology book (Holt *et al*., 1994).

Bacterial Identification by Using 16S rRNA Gene

Bacterial isolate was previously grown in Luria-Bertani broth media (LB) in room temperature for 18 hours (Victoria *et al.* 2009). Afterwards, 1 ml culture was centrifuged in 5000 g for 10 minutes. Supernatant was discarded and the pellet of cell used for the next step. DNA extraction step was done according to *DNeasy® QIAGEN* kit.

DNA extraction was then amplified by using polymerase chain reaction (PCR). Primer used was 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGCTACCTTGTTACGACTT-3') (Devereux & Wilkinson 2004). As much as 12.5 µl of dNTP *Go Taq® Green Master Mix (Promega),* 1 µl Primer 27f 10 µM, 1 µl Primer 1492r 10 µM, 2 µl DNA extract, and 8.5 µl of nuclease free water (NFW) were mixed to reach the total volume of 25 µl.

PCR thermal cycle was conducted by using *Applied Biosystem® GeneAmp® PCR System 9700.* Thermal cycle parameter used was according to modified Galkiewicz and Kellogg method (2008). The steps conducted were as followed: initial denaturation in 94^{o}C for 15 minutes (1 time), denaturation in 94 $^{\circ}$C for 1 minute (30 times), annealing in 54^{o}C for 1 minute (30 times), extension in 72 $^{\circ}$C for 2 minutes (30 times), final extension 72 $^{\circ}$C for 10 minutes (1 time), and storage done in 4 $^{\circ}$C temperature. As much as 5 µl of amplification result then used for electrophoresis analysis in 1% agarose gel, SBYR Safe 4 µl and pH 7 buffer TAE 1x. 5 µl marker 100 bp, negative control (without DNA extract) and DNA extract of each isolate were filled into the well. They were then examined by UV transilluminator. Purification of amplification result was done by ethanol method. 20 µl *HiDi formamide®* was then added and sequencing analysis was done by using *Applied Biosystem®* 3130 G*enetic Analyzer*. Sequencing result data was then compared with data acquired from Gen Bank with BLAST server (Basic Local Alignment Search Tool) method from National Center for Biotechnology Information (NCBI).

Tested of Phosphate Dissolving Ability

The selected of phosphate solubilising bacteria were used to test the ability of phosphate dissolving using soil samples. Each of soil samples was previously left to air dry then crushed with mortar and sifted with screen mesh 20. As much as 20 g soil were weighted then put in Erlenmeyer and added with 200 ml nutrient solution which consists of 400 mg glucose, 400 mg sucrose, and 75 mg NH_4NO_3 that were sterilized in 121 ^{o}C, 1 atm for 15 minutes. 1 ml of bacterial isolate was suspended in the media and then incubated for 2 weeks in room temperature (Illmer & Schinner 1995). The suspension was then centrifuged in 10000 rpm (*Hettich® Zentrifugen Mikro 200R*) at room temperature. The supernatant was measured using molybdate-vanadate method and the absorbance was measured by using visible light spectrophotometer (*Thermo Scientific® Genesys 20*) on 400 nm wavelength (International Organisation of Vine and Wine, 2015). The ability of the bacterial isolate to dissolve phosphate in the sample soil was measured by following equation:

$$\text{Index of } PO_4 \text{ dissolving ability} = \frac{[\,PO_4\,]_{end} - [\,PO_4\,]_{initial}}{[\,PO_4\,]_{initial}}$$

Result and Discussion

Soil Component Sampling and Analysis

The quarry was located in Palimanan Cirebon, West Java Province, Indonesia. In this research, samples taken from limestone quarry land are then continued to be called "soil". It was in line with the concept stated by Sposito (2008) that soil is the weathering process of rock however in this part it was very difficult to distinguish the borderline between soil and rock. Basically soil is different with rock weathering because if seen vertically (soil horizon), then the closer it is to the surface, there is water infiltration activity and living organism activity. Seen from chemical composition, soil is a part of an open system consists of multi component and biogeochemistry system which comprised of solid, liquid, and gas (Sposito, 2008). The pH of all soil samples were in the range alkaline between 8.8 to 9.7. In the soil, phosphate was generally bounded with calcium and unable to be absorb by plants. The soil sample of QC4 had pH of 9.7 and calcium concentration 10858.54 ppm (Table 1).

According to Jianbo *et al*. (2011), if increasing pH was occurred in soil, the solubility of Fe phosphate and Al phosphate will increase, however solubility of Ca phosphate will decreased. Phosphate usually precipitated by Ca, initially in dicalcium phosphate form which can be absorbed by plants. Afterwards, dicalcium phosphate will change into more stable form such as octocalcium phosphate and hidroxiapatite (HAP) which is hard to be absorbed by plant. Within calcareous rock, there are more than 50% HAP from an-organic P total. HAP compound will be easy to be soluble if pH decrease happens.

Table 1 The element component of soil samples from five locations in quarry Palimanan- Cirebon

Compound	Measurement	QC 1	QC 4	QC 5	QC 6	QC 7
Total Phosphate	mg/kg	2390.00	1190.00	1270.00	1060.00	1230.00
Mn	mg/kg	157.75	143.71	225.94	146.61	553.11
Ca	mg/kg	4370.88	10858.84	8803.47	6741.17	3656.55
Al	mg/kg	3426.31	1260.77	3772.17	2245.93	5417.71
Fe	mg/kg	880.83	1896.40	1617.81	1304.97	821.62
Mg	mg/kg	67.80	161.58	139.56	108.44	66.04
K	mg/kg	313.06	174.47	447.55	176.17	341.87
Na	mg/kg	25.67	13612.51	50.65	29.85	16.12
Cl	mg/kg	385.00	359.00	363.00	370.00	666.00
Water content	%	15.58	2.08	16.62	19.19	23.20
pH		9.5	9.7	8.8	9.2	9.6

Phosphate Solubilising Bacteria Isolation

Microbial exploration was done in area around the mining in order to get microbes which adapted on high calcareous condition and could survive at basic pH condition. Nutrient cycle in the soil especially P element is affected by the existence of soil microbes (Sposito 2008). If the existence of microbes within the soil disappeared, then nutrient would be hard to be absorbed by plant. There were totally 4 bacterial isolates could form halo-zone on Pikovskaya medium. QC5B.3 had the highest phosphate solubility index (Table 2).

Table2 The phosphate solubility index (SI) of 4 bacterial isolates

Isolate code	Colony Diameter (cm)	Halo-zone Diameter (cm)	Phosphate solubility index (SI)
QC5A.1	2.6	3.2	0.23
QC5B.1	2.3	2.9	0.26
QC5C.1	0.7	0.9	0.29
QC5B.3	0.7	1.0	0.43

Bacterial Identification Based on Biochemical Test and 16S rRNA Gene

All bacterial tested were categorized Gram negative bacteria, rod shapes and had specific of biochemical characteristic (Table 3). DNA extraction treatment followed the protocol of *DNeasy® QIAGEN* kit. After extraction, electrophoresis observation was conducted on agarose gel. Each produced 16S rRNA gen band ~1300 bp. The four purified isolates were then underwent sequencing stage, which then the result would undergo BLAST and being matched with Gen Bank from National Center for Biotechnology Information (NCBI) (Table 4).

Table 3. Biochemical characteristic of four bacterial isolates

No.	Characteristic Test	Bacterial Isolates			
		QC5A.1	QC5B.1	QC5C.1	QC5B.3
1.	Gram stain	-	-	-	-
2.	KOH 3%	B	B	B	B
3.	Shape	rod	rod	rod	Rod
4.	Motility	+	+	-	-
5.	Catalase	+	+	-	+
6.	Oxidase	-	-	-	-
7.	Glucose (acid; gas)	+	+	+	+
8.	H_2S	-	-	-	-
9.	Ornithine	+	+	+	+
10.	Oxidative/fermentative (O/F)	O	O	O/F	O
11.	*Methyl Red*	-	-	-	-
12.	*Voges-Proskauer*	+	+	-	+
13.	Citrate (*Simmons*)	+	+	+	+
14.	Urea hydrolysis	-	-	-	-
15.	Arginine dihidrolase	+	+	-	-
16.	Gelatin Hydrolysis	+	+	-	-
17.	L-Arabinosa	+	+	+	+
18.	Sucrose	-	-	-	-
19.	Nitrate (reduction)	-	-	+	+
20.	Indol	-	-	-	-

Ket: B = slime, TB = not slime, O = oxidative, F = fermentative

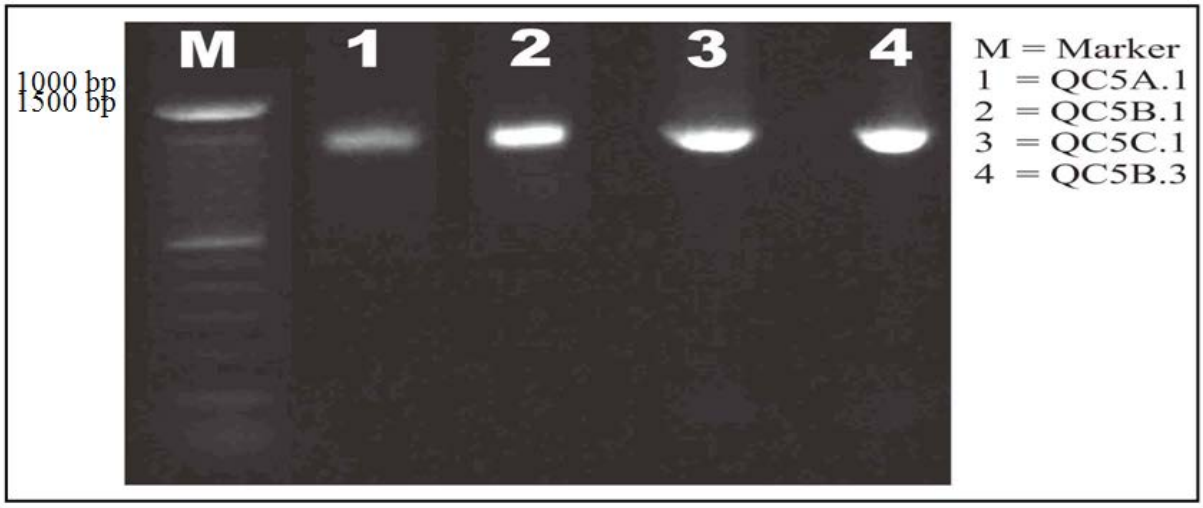

Figure 2. Amplification results of 16SrRNA gene for phosphate solubilizing bacteria

Table 4 The result of 16S rRNA gen analysis by using *BLAST NCBI* program

Isolates	Species Bacteria	*Gen Bank Accession*	% Identity
QC5A.1	*Pseudomonas mosselii*	DQ095881.1	99 %
QC5B.1	*Pseudomonas mosselii*	DQ095881.1	99 %
QC5C.1	*Pantoea ananatis*	FJ796221.1	99 %
QC5B.3	*Pseudomonas oryzihabitans*	KF192618.1	100 %

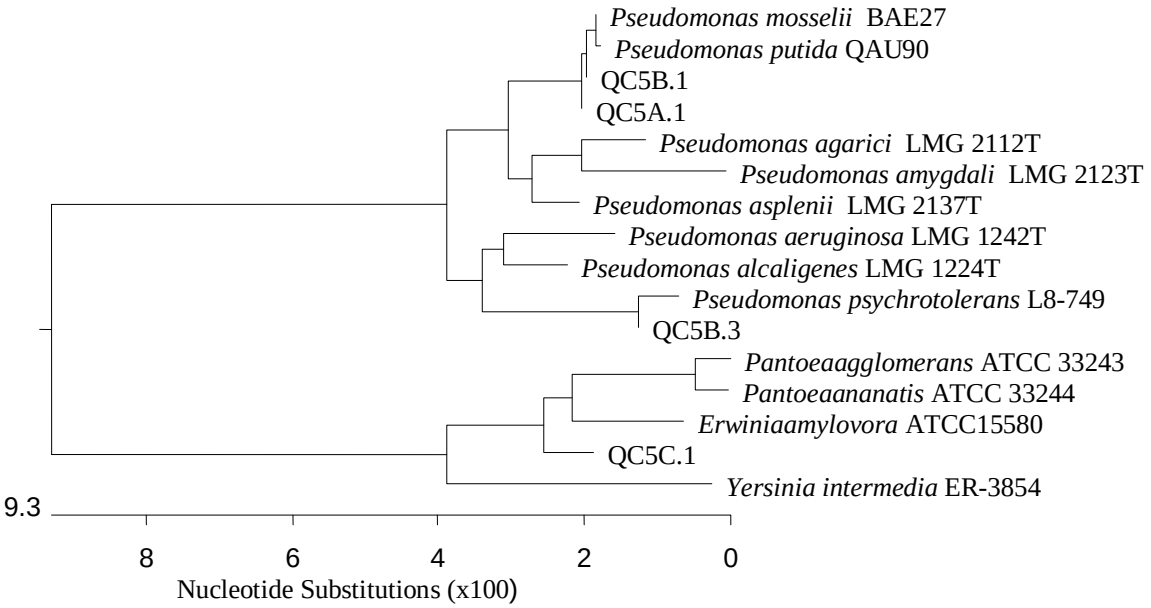

Figure 3. Phylogenetic tree relationship between 4 isolates (Kwon *et al*. 1997; Godfrey *et al*. 2001)

Determination of Phosphate Diluting Ability of Bacteria

The index ability of dissolving phosphate in soil samples was calculated by comparison of final phosphate concentration to initial phosphate concentration between 1.63 (QC5A.1 on QC7) and 3.42 (QC5B.1 on QC1). All isolates were capable of dissolving phosphate higher against QC1 compared too ther soil samples. The variety of soluble phosphate content depend on the type of bacteria and the type of soil tested (Figure 4).

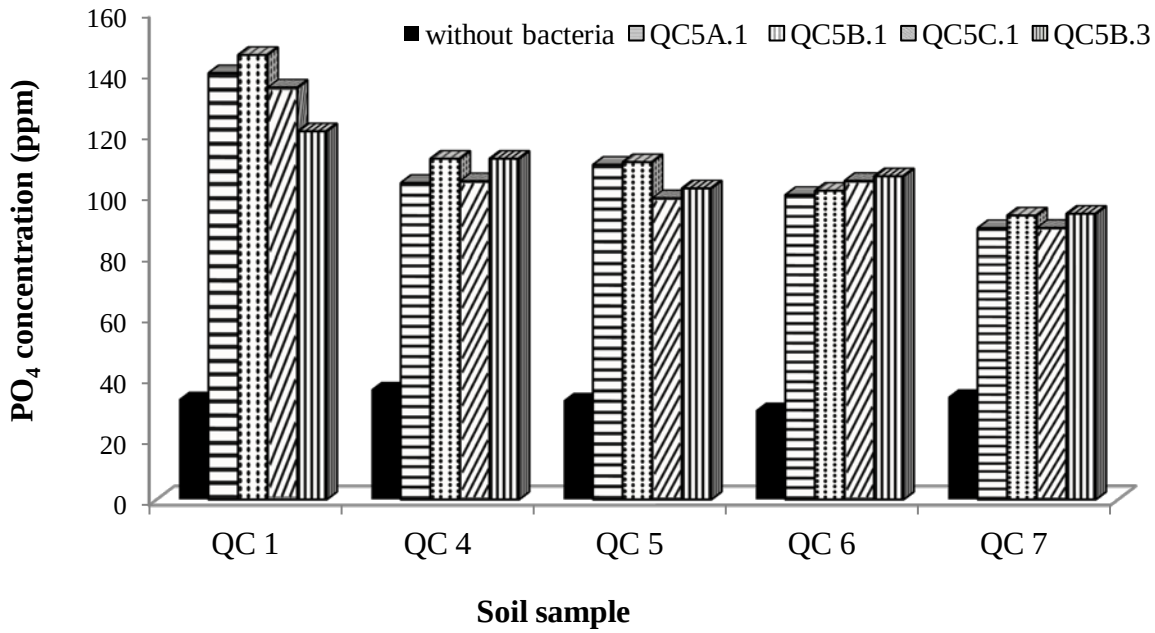

Figure 4. Bacterial phosphate diluting ability of 5 isolates using soil samples

Conclusion

Four bacterial isolates which isolated from Palimanan - Cirebon limestone quarry area could dissolve $Ca_3(PO_4)_2$ in Pikovskaya media. Phosphate dissolving index of the isolates are in range 0.23 – 0.43. The isolates were identified by using 16S rRNA gen identification and showed similar with *Pseudomonas mosselii* (QC5A.1), *Pseudomonas mosselii* (QC5B.1), *Pantoea ananatis* (QC5C.1), and

Pseudomonas oryzihabitans (QC5B.3), respectively. Phosphate dissolving ability index on 5 soil sample taken from different limestone quarry showed increasing of phosphate dilution between 1.63 until 3.42. All isolates were able to grow and adapt on alkaline and calcareous condition.

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