



## Characterization of phosphate solubilizing bacteria and nitrogen fixing bacteria from limestone mining region

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### ABSTRACT

**Aims:** Phosphate and nitrogen are major macronutrients needed by plants. Phosphates in the soil are present in the organic and inorganic form. The amounts of phosphate and nitrogen in marginal soil can be increased by plant growth promoting rhizobacteria (PGPR). The aim of this study was to isolate and characterize phosphate solubilizing bacteria which has ability to fix nitrogen from the soil around limestone mining area.

**Methodology and results:** There were 22 isolates that could solubilize phosphate and fix nitrogen. There were 9 isolates that could not cause hypersensitivity symptoms (necrotic) on tobacco leaf. Quantitative assay of phosphate solubilizing was done by colorimetric method. Quantitative assay of phosphate showed that isolate GPC1.7 had the highest phosphate solubilizing activity on Pikovskaya broth (450 mg/L) on the 6<sup>th</sup> and 7<sup>th</sup> day of incubation whereas isolate GPA2.2 had the highest nitrogen fixing activity (0.162 ppm/h), measured with Acetylene Reduction Assay whereas nitrogenase activity of GPC1.7 was unidentified. Isolate GPA2.1 and GPA2.2 were Gram negative bacteria whereas isolate GPC1.7 was Gram positive bacteria. Identification based on 16S rRNA gene showed that GPA2.1 was closely related to *Pseudomonas psychrotolerans*, GPA2.2 was closely related to *Stenotrophomonas maltophilia*, GPC1.7 was closely related to *Bacillus megaterium* and *B. aryabhatai*.

**Conclusion, significance and impact of study:** Phosphate solubilizing bacteria and nitrogen fixing bacteria isolate could be further used for revegetation process of the ex-limestone mining area.

**Keywords:** 16S rRNA, nitrogen fixing bacteria, limestone soil, phosphate solubilizing bacteria

### INTRODUCTION

Phosphate and nitrogen are macronutrient that is needed by plants, especially for various protein formations. Most of soil nitrogen (90-95%) is bound to organic materials, whereas the rest is used by plants in inorganic forms (NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup>) (Murphy, 2014). Soil phosphates are present in organic and inorganic forms. Both are difficult to soluble, therefore the availability for organism especially plants are limited (Saraswati *et al.*, 2007). Chemical contents, including nitrogen and phosphate, in marginal or critical land are lower than in fertile soil (Suharta, 2010). It was recorded that critical land in Indonesia reached 27,296,000 hectares in 2012 (Statistic Center Institution, 2014). Critical dry land that was temporarily unused in Indonesia on the same year was recorded as much as 14,252,383 hectares (Cakrabawa *et al.*, 2013). The ex-limestone mining soils physically, chemically and biologically has low fertility for agriculture activities (Prayudyarningsih, 2014), that is categorized as marginal land.

Marginal soils such as ex-limestone mining soil need the revegetation. The use of NPK (nitrogen, phosphorus, and potassium) fertilizer was one of the alternatives. NPK fertilizer could increase nitrogen concentration in soil (Mazzoncini *et al.*, 2011). But, the excessive use could cause negative effects for plants such as root growth inhibition (Herdiana *et al.*, 2008), and for environment, that is causing pollution and fast depletion of other nutrients such as S, Ca, Mg, Zn, and Cu (Las *et al.*, 2006). Therefore, environmental friendly and specific agents that can fix nitrogen and solubilize inorganic phosphate from soil are needed. One of biological agents that can fix nitrogen and solubilize phosphate is from plant growth promoting rhizobacteria (PGPR) group.

Plant growth promoting rhizobacteria is a group of bacteria that live around roots area and able to produce plant growth promoting substances, such as hormone and enzyme. Some rhizobacteria group are able to fix nitrogen and live symbiotically with roots, forms nodul, such as *Bradyrhizobium japonicum* or free living as *Azotobacter* sp.

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(Moat *et al.*, 2002). Phosphate solubilizing bacteria can convert insoluble organic and inorganic phosphate to soluble form that beneficial for plants. Solubilizing activity of *Arthrobacter ureafaciens*, *Phyllobacterium myrsinacearum*, *Rhodobacter erythropolis*, and *Delfia* sp. were associated with organic acid releasing and pH medium decreasing (Chen *et al.*, 2006).

Nitrogen fixing and phosphate solubilizing PGPR bacteria can be isolated from mining area soil. Mursyida *et al.* (2015) could isolate phosphate solubilizing bacteria from limestone mining soil, Palimanan Quarry. Those are *Burkholderia cepacia*, *Serratia marcescens*, and *Pseudomonas putida*. Other bacteria from *Azotobacter* genus had been isolated from phosphate mining (Reyes *et al.*, 2006). Generally, natural phosphate deposit can be found around regions that contain lime (Sastramihardja *et al.*, 2009). One of limestone mining, PT Indocement is located in Cirebon, West Java. Revegetation is needed in the open ex-mining limestone areas there. Various bacteria species are potential as biofertilizer that can be used for revegetation process. PGPR bacteria potential as nitrogen fixer and phosphate solubilizer from PT Indocement limestone mining soil need to be further explored, that can be useful for revegetation process. The aim of the study is to isolate and characterize phosphate solubilizing bacteria which has ability to fix nitrogen from the soil around limestone mining area.

## MATERIALS AND METHODS

### Materials

Soil samples were obtained from Blindis Mountain, around limestone mining area of PT Indocement, Palimanan, Cirebon (S 06° 33.215 E 106° 41.550).

### Phosphate solubilizing bacteria isolation

Isolation was done by dilution of 1 g soil sample in NaCl 0.85% until  $10^{-4}$  dilution factor. Serial dilution method was done following Hadioetomo (1993). One milliliter for each dilution was spread on Pikovskaya media (Nautiyal, 1999). It was stored at 28 °C for 7 days incubation time. The colony that formed halo zone was purified and sub-cultured on Pikovskaya agar. Morphological characteristics of each colony were observed following Holt *et al.* (1994).

### Nitrogen fixing bacteria selection

Phosphate solubilizing isolates were grown on nitrogen free bromothymol blue (NfB) media (Okon *et al.*, 1977), incubated at 28 °C for 7 days incubation time. Selected isolates were also grown on Congo Red Agar (CRA). The composition of CRA medium was same as NfB, added with 0.0025% Congo Red (10 mL/L), 0.05 g yeast extract, and 15 g of agar (Cáceres, 1982). Isolate that couldn't grow on CRA medium, but could grow on NfB medium was grown on Yeast Mannitol Agar (YMA), added with 0.0025% Congo red (Situmorang *et al.*, 2009).

### Qualitative estimation of phosphate solubilization

Qualitative estimation was done by dot method using sterile tooth stick on Pikovskaya agar and was incubated in room temperature for 7 days. Solubility index (SI) was measured by subtracting the value of halo zone diameter toward the value of colony diameter. The resulted value divided by colony diameter (Mursyida *et al.*, 2015).

### Quantitative estimation of phosphate solubilization

Quantitative estimation of phosphate solubilization was done based on colorimetric assay. Selected isolates (GPA2.1, GPA2.2 and GPC1.7) were cultivated in 100 mL Pikovskaya broth, incubated in shaking incubator for 7 days at 37 °C. Every 24 h, 1.5 mL of each culture was centrifuged at 10600  $\times$ g for 10 min. Each 1 mL were reacted with color forming reagents (2.5 mL sodium molybdate 2.5% and 1 mL hydrazine sulfate 0.3%), which formulation was made by following Lynn *et al.* (2013).

### Nitrogenase activity assay

Nitrogenase activity assay was measured by Acetylene Reduction Assay (ARA) method using gas chromatography instrument (Gibson and Turner, 1980). The selected isolates were grown overnight in NfB medium at 30 °C. Each 0.5 mL culture was inoculated to 5 mL NfB that was placed in 25 mL tube (Neilson and Sparell, 1976), incubated for five days at 30 °C (Harca, 2015). After five days of incubation, the tube was covered by rubber cork and parafilm paper. The ARA measurement were done by dropping the air in the tube out, used 1 mL sterile syringe, then the acetylene gas ( $C_2H_2$ ) was injected into tube with the same volume as the dropped out air. After 2 h of incubation, ethylene gas on in the tube was measured by using gas chromatography instrument.

### Hypersensitivity assay

One mL bacterial isolate that was grown in nutrient broth (NB) (cell density  $\pm 10^8$  cell/mL) was injected on the below surface of tobacco leaf by using syringe (without needle) (Zou *et al.*, 2006). *Pseudomonas syringae* was used as positive control whereas *Bacillus cereus* and sterile media were used as negative control. Hypersensitivity respond was observed at 18 h after injection time (Wahyudi *et al.*, 2011).

### Antagonist transisolate assay

The selected isolates were grown in 10 mL NfB medium and incubated in shaker incubator for 24 h at room temperature. Each 400  $\mu$ L of culture was mixed in 40 mL warm nutrient agar (NA), and then was poured into sterile petri disc. Wells were formed after the media was compacted by using sterile straw, as the place for other isolates and controls. Each well was filled with 15  $\mu$ L culture. Sterile distilled water was used as negative

control whereas kanamycin (1000 mg/L) was used as positive control.

### Identification based on 16S rRNA gene

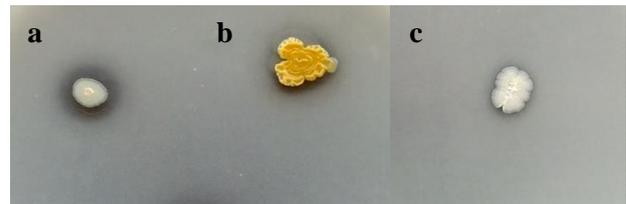
The DNA of selected isolates was isolated following the Presto™ Mini gDNA Bacteria Kit protocol. The 16S rRNA gene was amplified by Polymerase Chain Reaction (PCR), using specific primer for bacteria, 63f (5'-CAGGCCTAACACATGCAAGTC-3') and 1387r (5'-GGGCGGWGT-GTACAAGGC-3') (Marchesi *et al.*, 1998). The mix PCR was made up to 10 µL, consists of 5 µL GoTaq Green (Promega), 0.5 µL 63f primer, 0.5 µL 1387r primer, 1 µL DNA template and 3 µL ddH<sub>2</sub>O. The mix PCR then was placed into PCR machine by following condition: pre-denaturation (94 °C, 5 min), denaturation (92 °C, 30 min), annealing (55 °C, 30 sec), elongation (72 °C, 1 min), and post elongation (72 °C, 5 min). PCR was made up to 30 cycles. The amplified DNA then was electroforated at 80 V for 30 min to see the pureness. The amplified DNA samples were sent to sequencing service provider. The DNA sequence was aligned with GenBank data by using Basic Local Alignment Search Tool-Nucleotide (BLASTN) from National Center for Biotechnology Information (NCBI). Phylogenetic tree were made by using MEGA6 software (Tamura, 2013).

## RESULTS

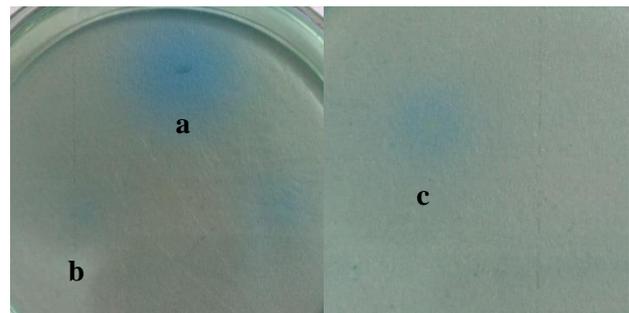
### Isolation of phosphate solubilizing and nitrogen fixing bacteria

There were 44 phosphate solubilizing bacterial isolates were found from 15 soil samples around limestone mining area, Blindis Mountain, Cirebon. The isolate that formed clear zone around the colony on Pikovskaya media was known as phosphate solubilizing bacteria (Figure 1). The solubility index of all strain was measured about 0.125 to 2.375. All phosphate solubilizing isolates were grown on NfB and CRA. There were 22 phosphate solubilizing isolates were grown on NfB and turned the media into blue color, whereas 39 isolates were grown on CRA, 19 isolates were absorbed Congo red. Generally, bacteria could fix nitrogen with available source such in CRA. Bacteria that could not grow on CRA but could grow on NfB were grown on YMA supplemented with Congo red 0.0025%.

Colonies that grew on NfB showed that the bacteria could fix free nitrogen because media did not content the nitrogen source. Nitrogen fixing bacteria could change the color of NfB media from green to blue (Figure 2). Colonies that could absorb Congo red were indicated as nitrogen free fixing bacteria. Of all isolates, 10 isolates were indicated as symbiotic living microorganisms whereas 12 others were indicated as nitrogen free fixing bacteria.



**Figure 1:** The selected isolate colony of GPA2.2 (a) GPA2.1 (b) and GPC1.7 (c) on Pikovskaya agar after 7 days of incubation.



**Figure 2:** The selected isolate colony of GPA2.1 (a), GPA2.2 (b) and GPC1.7 (c) on NfB solid media after 3 days of incubation.

### Quantitative estimation of phosphate solubilizing bacteria

There were three isolates chosen, GPA2.1, GPA2.2 and GPC1.7, based on their phosphate solubility index, nitrogen fixing ability and growth stability (Table 1). The maximum phosphate solubility on Pikovskaya broth for isolate GPA2.1 and GPA2.2 were observed on 5<sup>th</sup> day of incubation, 112.3 mg/L and 69.3 mg/L, respectively. The highest phosphate solubility was observed for isolate GPC1.7 (450 mg/L) on 6<sup>th</sup> and 7<sup>th</sup> day of incubation (Figure 3).

### Nitrogenase activity

Nitrogenase activity was observed for isolates GPA2.1 and GPA2.2, whereas isolate GPC1.7 could not be measured or unidentified. Isolate GPA2.2 had the highest nitrogenase activity per hour (Table 2). Nitrogenase of GPC1.7 might not be measured because its concentration was too low or there was no nitrogenase activity.

### Hypersensitivity assay towards tobacco leaf

Of all isolates, 9 isolates showed negative hypersensitivity symptoms on tobacco leaf after 48 h of incubation, based on negative controls, sterilized aquadest, media and *B. cereus*. *Pseudomonas syringae* as positive control showed causing yellow-brownish spot on tobacco leaf.

**Antagonistic assay among bacterial isolates**

All isolates did not show antagonistic reaction to each other, compared with positive control, kanamycin (1 mg/mL). The clear zone that appeared was shown as growth inhibition area of tested bacteria.

**Identification of isolates**

The selected isolates showed different characters of colony based on shape, margin, elevation and color (Table 3). Based on Gram staining, the bacterial isolate GPA2.1 and GPA2.2 were categorized as Gram negative

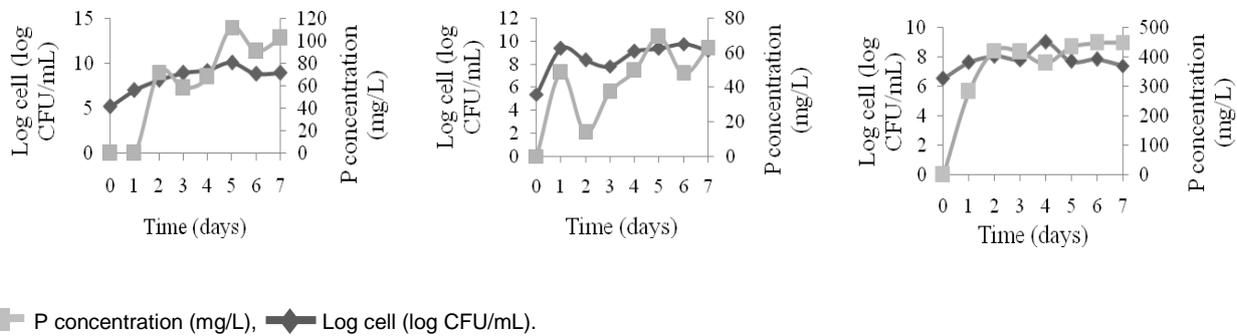
whereas GPC1.7 was Gram positive. The cell shape of all isolates was rods (Figure 4).

Based on the molecular identification by using 16S rRNA gene, each isolate produced amplicon 1300 bp length. The isolate GPA2.1 was closely related to *Pseudomonas psychrotolerans* with similarity level 99%, isolate GPA2.2 were closely related to *Sternotrophomonas maltophilia* with similarity level 89%, and GPC1.7 were closely related to *Bacillus megaterium* and *B. aryabhatai* with similarity level 100%, respectively (Figure 5). The access number and e- value of bacteria on phylogenetic tree was shown on Table 4.

**Table 1:** Growth characteristics of nine selected isolates.

Isolate code	Gram	Phosphate Solubility Index	Growth on media			Hypersensitivity to tobacco leaves
			NfB	CRA	YMA	
GPA2.1	Negative	0.304	+++***	+++	0	Negative
GPA2.2	Negative	0.263	+*	++**	0	Negative
GPC1.3	Positive	0.444	+++	-	+++	Negative
GPC1.7	Positive	0.278	++**	+++	0	Negative
GPC3.7	Negative	2.375	++	+++	0	Negative
GPC4.2	Negative	0.714	+	+	0	Negative
GPC4.3	Positive	0.206	++	+++	0	Negative
GPC4.6	Negative	0.250	++	+++	0	Negative
GPC4.11	Negative	0.229	+	+++	0	Negative

Information: \*, small colony size; \*\*, moderate colony size; \*\*\*, big colony size; -, not growing bacteria; 0, isolate was not grown on the media.



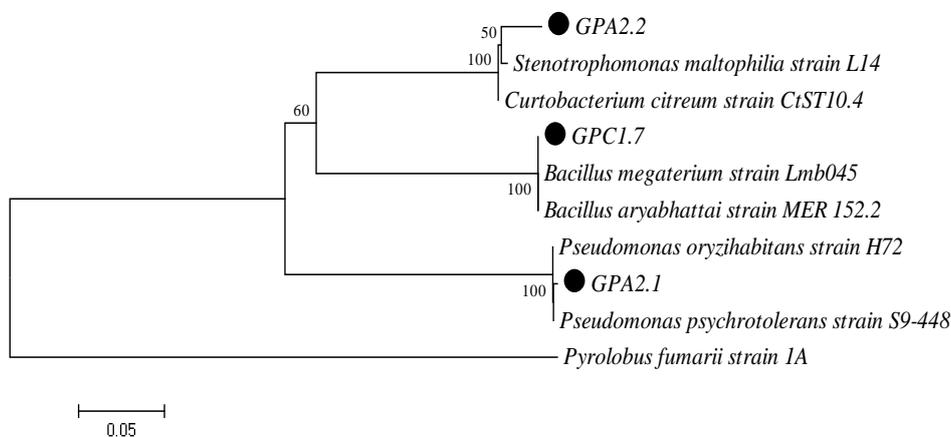
**Figure 3:** Amount of cell and phosphate solubility concentration of 4 selected isolates GPA2.1 (a), GPA2.2 (b) and GPC1.7 (c) for 7 days observation.

**Table 2:** Nitrogenase activity of selected isolates.

Isolate code	Ethylene concentration (ppm/h)
GPA2.1	0.064
GPA2.2	0.162
GPC1.7	Unidentified



**Figure 4:** Gram staining of isolate GPA2.1 (a) GPA2.2 (b) GPC3.7 (c) and GPC1.7 (d) observed at 1000× magnification. The line on the lower corner had measurement of 1 µm.



**Figure 5:** Phylogenetic tree construction of four selected isolate based on 16S rRNA sequence.

## DISCUSSION

Plant growth promoting rhizobacteria (PGPR) is a group of bacteria that colonize around plant root or rhizosphere. Some of PGPR are able to solubilize phosphate and fix nitrogen that can be further use by plant. Phosphate solubilizing and nitrogen fixing bacteria can be isolated from any kind of soils and habitats. Chen *et al.* (2006) isolated phosphate solubilizing bacteria from subtropic soil in Taiwan. Nitrogen fixing bacteria that are able to solubilize phosphate could be isolated from some species of lichens, namely *Canoparmelia caroliniana*, *C. crozalsiana*, *C. texana*, *Parmotrema sancti-angeli* and *P. tinctorum* (Liba *et al.*, 2006). Siddike *et al.* (2010) isolated phosphate solubilizing bacteria and nitrogen fixing halotolerant bacteria from soil around coastal area. Phosphate solubilizing psychrotolerant bacteria from extreme region such as Antarctic had also been isolated (Selbmann *et al.*, 2010).

The soil of limestone mining area chemically has high phosphate total content and low nitrogen total content. Based on Eviati and Sulaeman (2009), the soil sample from around limestone mining region was grouped as sandy loam. The soil is not fertile for plant because the high content of calcium could easily bind the phosphate, causing the limitation of phosphate availability for plant.

Therefore, on restoration, rehabilitation or reclamation process of limestone ex-mining area we need the role of phosphate solubilizing and nitrogen fixing PGPR. The macronutrient phosphate and nitrogen are needed by plant for growing and developing, including root, stem, branch and leaf growth, flower forming, fruit and seed ripening, cell wall arrangement, and chlorophyll synthesis (Arancon *et al.*, 2004; Khozin-Goldberg and Cohen, 2006; Prasetyo and Suriadikarta, 2006).

Phosphate solubilizing bacteria could be isolated by using selected media, which is the Pikovskaya media. Phosphate solubilizing bacteria isolate that grow in Pikovskaya media is able to form clear zone around the growth colony. Clear zone forming indicates the phosphate solubilization activity and acid forming by bacteria (Tripti *et al.*, 2012). Tricalcium phosphate ( $\text{Ca}_3\text{PO}_4$ ) in media could be raveled and absorb by plant on ion form ( $\text{Pi}$ ,  $\text{HPO}_4^{2-}$ ,  $\text{H}_2\text{PO}_4^-$ ) (Rodriguez and Fraga 1999). Some bacteria were able to solubilize different source of phosphate such as hydroxyapatite and phosphate stone (Kim *et al.*, 1997; Khan *et al.*, 2007).

Nitrogen fixing bacteria was selected by three media, which were the NfB, CRA and YMA. NfB was a selected medium for the growth of nitrogen fixing bacteria because the media did not contain the nitrogen source. A number of researches used NfB as selected media for nitrogen

free living bacteria (Jolly *et al.*, 2010; Kanimozhi and Paneerselvam, 2011; Isti'annah, 2014). CRA was used on this research to distinguish nitrogen fixing bacteria that live freely and symbiotically with plant root. Congo red in CRA media could distinguish rhizobia group with nitrogen free living bacteria *Azospirillum* (Cáceres, 1982). Congo red stains amyloid and bacteria cellulose, also interact with  $\beta$ -D-glucan and polysaccharide on its rhizobia group capsule (Kneen and Larue, 1983). YMA plus Congo red medium has the same function as CRA. Generally, YMA medium is used for cultivating nitrogen fixing symbiotic rhizobia such as *Bradhyrhizobium japonicum* that could not absorb Congo red (Mubarik and Sunatmo, 2014). The used of NfB, CRA and YMA medium on selection process could give us preliminary information about the ability of nitrogen fixation by bacteria.

The ability of phosphate solubilization, was quantitatively observed for three selected isolates, GPA2.1, GPA2.2 and GPC1.7, were grown in Pikovskaya broth medium. Based on phosphate solubility curve (Figure 3), there was no correlation between cell growth (log cell) and the amount of soluble phosphate. Phosphate solubilization of isolate GPA2.1, GPA2.2, and GPC1.7 were fluctuated on 7 days of observation. The fluctuating concentration of soluble phosphate in broth media could happen because of the precipitation phosphate from organic metabolic and or the formation of organophosphate compound along with organic acid secretion. The organic acid then could be further used as energy or nutrient source. This condition could be repeated several times in a culture (Illmer and Schinner, 1992; Lugtenberg and Kamilova, 2009). Rodriguez and Fraga (1999) gave the explanation about the fluctuation of soluble phosphate, related with the uptake of soluble phosphate as energy or nutrient source. If the rate of soluble phosphate uptake is higher than phosphate solubilization rate, the decreasing of concentration might have been observed. In contrast, when the rate of soluble phosphate uptake decreases (for instance because of bacteria stop growing or at growth condition before stationery phase), the amount of soluble phosphate in media increases.

Isolate GPC1.7 had the highest phosphate solubilization on Pikovskaya broth (450 mg/L) whereas the phosphate solubility index qualitatively was 0.278. Phosphate solubility index could not determine whether a bacterium is strong or weak phosphate solubilizer. There was no correlation between bacteria that solubilize phosphate on Pikovskaya agar and in Pikovskaya broth medium. Some researchers had reported the same condition (Rodriguez and Fraga, 1999; Alikhani *et al.*, 2006; Mursyida *et al.*, 2015). Qualitative assay only used in the preliminary of study to describe bacteria phosphate solubilization ability.

The activity of nitrogen fixing bacteria will be unorganized without nitrogenase enzyme. From preliminary screening, we assumed that all four bacteria, qualitatively could fix nitrogen. Nitrogenase activity of all four isolates was measured by Acetylene Reduction Assay (ARA). Acetylene ( $C_2H_2$ ) in ARA method was used

as alternative substrate. Nitrogenase complex reduces the three bond of  $N_2$  to ammonia form. Nitrogenase activity is measured due to ethylene gas ( $C_2H_4$ ) production. The comparison between the reduction of  $N_2$  and acetylene ( $N_2:C_2H_4$ ) by nitrogenase is 3:1 (Mubarik and Sunatmo, Yu 2014). Isolate GPA2.2 had the highest nitrogenase activity (0.162 ppm/h). The nitrogenase activity was higher than *Beijerinckia fluminensis* (0.094 ppm/h) that was isolated from soil around Jambi forest (Harca 2015). Pinto-Tomás *et al.* (2009) reported that the average of nitrogenase activity from bacteria that live symbiotically with cutting-leaf ant from genus *Atta* and *Acromyrmex* was 1.03 nmol ethylene per day.

Nitrogenase activity was unidentified for isolate GPC1.7, whereas qualitatively it grew pretty well on NfB medium (Table 1). As it was stated before that the way of colony growing on selective media could not be correlated to quantitatively assay such as ARA. Nitrogenase is the important key of  $N_2$  transformation to ammonia ( $NH_3$ ). Nitrogenase is encoded by a set of operon, including regulator genes (such as *nifL* dan *nifA*), structural genes (such as *nifH*, *nifD* and *nifK*) and other supporting genes. The *nifH* gene regulates the Fe protein whereas *nifD* and *nifK* genes regulate Mo-Fe protein (Cheng, 2008).

The phylogenetic tree showed that isolate GPA2.1 was *P. psychrotolerans* with similarity level 99%, isolate GPA2.2 were closely related to *S. maltophilia* with similarity level 89%, and isolate GPC1.7 were closely related to *B. megaterium* and *B. aryabhattai* with similarity level 100% (Figure 5). *Pseudomonas psychrotolerans* is bacteria that could adapt to cold weather ( $\leq 15^\circ C$ ) and was reported as potential phosphate solubilizer (Subramanian *et al.*, 2011; Han *et al.*, 2013). Psychrotolerant bacteria from the same genus, *P. lurida* also had been reported as phosphate solubilizer (Selvakumar *et al.*, 2011). *Sternotrophomas maltophilia* characteristically had the same color with isolate GPA2.2 which is white to pale yellow, but sometime is brownish because of the secondary chemical reaction among extracellular products (Denton and Kerr, 1998). The used of inoculant *S. maltophilia* could increase soluble phosphate uptake and nitrogen fixation of *Juglans siggilata* (Yu *et al.*, 2011). Some of *Azotobacter* genus and *B. megaterium* had been reported as they could solubilize phosphate (Elkoca *et al.*, 2008; Ogut *et al.*, 2010). Liba *et al.* (2006) reported that chemoorganotrophic bacteria *S. maltophilia* that was isolated from lichen could freely fix nitrogen. *Bacillus aryabhattai* had been known as phosphate solubilizer. The species had also been reported as zinc solubilizer and plant growth supporter under drought (Kavamura *et al.*, 2013; Ramesh *et al.*, 2014).

## CONCLUSION

Phosphate solubilizing bacteria that characteristically could fix nitrogen was isolated from limestone ex-mining area soil in Cirebon. The GPC1.7 bacteria isolate had the highest phosphate solubilizing activity on Pikovskaya broth (450 mg/L) on the 6<sup>th</sup> and 7<sup>th</sup> day of incubation

whereas isolate GPA2.2 had the highest nitrogen fixing activity (0.162 ppm/h). Isolate GPA2.1 and GPA2.2 were Gram negative bacteria whereas isolate GPC1.7 were Gram positive bacteria. Identification based on 16S rRNA gene showed that GPA2.1 was closely related to *P. oryzae*, GPA2.2 was closely related to *S. maltophilia*, GPC1.7 was closely related to *B. megaterium* and *B. aryabhatai*. All three bacteria isolates did not cause hypersensitivity reaction toward tobacco leaf and antagonistic reaction among isolates.

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