Characterization and Identification of Phosphate Solubilizing Bacteria Isolate GPC3.7 from Limestone Mining Region

D Fitriyanti¹, N R Mubarik¹ and A Tjahjoleksono¹

¹ Department of Biology, Bogor Agricultural University, INDONESIA.

E-mail: nrachmania@ipb.ac.id

Abstract. Phosphate (P) are one of major macronutrients needed by plants. P in the soil are present in the organic and inorganic form. The amounts of P in marginal soil can be increased with plant growth promoting rhizobacteria (PGPR). The aim of this study was to characterize and identify P solubilizing bacteria (PSB) isolate GPC3.7 that characteristically could fix N from the soil around limestone mining area. There were 44 PSB isolates found from 15 soil samples around limestone mining area, Blindis mountain, Cirebon. The solubility index of all strain were measured about 0.125 to 2.375 on Pikovskaya media. There were 22 PSB isolates were grown on N-free bromothymol blue (NfB) medium and 19 isolates were grown on Congo Red Agar (CRA) medium. Only 10 isolates were indicated as symbiotic living microorganisms whereas 12 others were categorized as N-free fixing bacteria. Isolate GPC3.7 was chosen to be further observed, based on its P solubility index, N-fixing ability and growth stability. Phosphate quantitative estimation assay of isolate GPC3.7 was unmeasured. The P soluble concentration of GPC3.7 might be lower than 1 mg/L. The colony of GPC3.7 morphologically had round shape, entire margin, raised elevation and white color. Isolate GPC3.7 was Gram negative bacteria with coccus cell shape. Based on 16S rRNA gene, GPC3.7 was closely relative to Acinetobacter baumannii.

1. Introduction
Phosphate (P) and nitrogen (N) are macronutrient that is needed by plants, especially for various protein formation. Most of soil nitrogen is bound to organic materials, whereas the rest is used by plants in inorganic forms (NO₃⁻ and NH₄⁺) [1]. Both organic and inorganic forms of soil phosphate are difficult to soluble, therefore the availability for organism especially plants are limited [2]. Chemical contents, including nitrogen and phosphate, in marginal or critical soil are lower than in fertile soil [3]. Statistics Institution of Indonesia [4] stated that critical land in Indonesia reached 27,296,000 hectares in 2012 whereas critical dry land that temporary unused in Indonesia on the same year was recorded as much as 14,252,383 hectares [5]. As marginal land, the ex-limestone mining soils physically, chemically and biologically has low fertility for agriculture activities [6].

Revegetation is needed for marginal soil such as ex-limestone mining soil. The use of NPK (nitrogen, phosphorus, and potassium) fertilizer was one of the alternatives most used. NPK fertilizer could increase nitrogen concentration in soil [7]. However, the excessive use could cause negative effects for plants such as root growth inhibition [8], and for environment, that is causing pollution and fast depletion of other nutrients (S, Ca, Mg, Zn, and Cu) [9]. Therefore, a specific and environmental friendly agent 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.

PGPR live around roots area and are 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 *Bradyrhizium japonicum* or free living as *Azotobacter* sp. [10]. Phosphate solubilizing bacteria can convert insoluble phosphate to soluble form that beneficial for plants. Phosphate solubilizing activity of *Arthrobacter ureafaciens* and *Delfia* sp. were associated with organic acid releasing and pH medium decreasing [11].

Nitrogen fixing and phosphate solubilizing bacteria can be isolated from mining area soil. Mursyida *et al.* [12] found phosphate solubilizing bacteria from limestone mining soil, Palimanan Quarry. Those are *Bulbholderia cepacia*, *Serratia marcescens* and *Pseudomonas putida*. Other bacteria from *Azotobacter* genus had been isolated from phosphate rock mining [13]. Natural phosphate deposit can be found around regions that contain abundant of lime [14]. One of limestone mining, PT Indocement is located in Cirebon, West Java. Revegetation is needed in the open ex-mining limestone areas there. PGPR potential bacteria as nitrogen fixer and phosphate solubilizer from PT Indocement limestone mining soil need to be further explored. One of the potential isolate coded as isolate GPC3.7. The aim of this study was to characterize and identify P solubilizing bacteria isolate GPC3.7 that characteristically could fix N from the soil round limestone mining area.

2. Material and Methods

2.1. Materials

Soil samples were obtained from Blindis mountain, around limestone mining area of PT Indocement, Palimanan, Cirebon.

2.2. Methods

2.2.1. Phosphate Solubilizing Bacteria Isolation. One gram of each soil sample was diluted in NaCl 0.85% solution until $10^{-4}$ dilution factor [15]. One mililiter of each dilution was spread on Pikovskaya media [16] and stored at 28 °C for 7 days incubation time. The colony that formed halo zone was purified and subcultured on Pikovskaya agar. Morphological characteristics of each colony were observed following Holt *et al.* [17].

2.2.2. Nitrogen Fixing Bacteria Selection. Each phosphate solubilizing isolates was grown on nitrogen free bromothymol blue (NfB) media [18], incubated at 28 °C for 7 days incubation time. Selected isolates were also grown on Congo Red Agar (CRA) [19]. 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 [20].

2.2.3. Qualitative Estimation on Phosphate Solubilization. Dot method was applied on qualitative estimation, 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 [12].

2.2.4. Quantitative Estimation of Phosphate Solubilization. Isolate GPC3.7 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 reagents consists of 2.5 mL sodium molybdate 2.5% and 1 mL hydrazine sulfate 0.3%. Formulation was made by following Lynn *et al.* [21].
2.2.5. **Nitrogenase Activity Assay.** Nitrogenase activity assay was measured by Acetylene Reduction Assay (ARA) method using gas chromatography instrument [22]. Isolate GPC3.7 was grown overnight in NfB medium at 30 °C. As much as 0.5 mL culture volume was inoculated to 5 mL NfB that was placed in 25 mL tube [23], incubated for five days at 30 °C [24]. The tube was covered by rubber cork and parafilm paper after 5 days of incubation. The ARA measurement were done by dropping the air in the tube out, used 1 mL sterile syringe, then the acetylene gas (C₂H₂) was injected into tube with the same volume as the dropped out air. After 2 hours of incubation, ethylene gas in the tube was measured, using gas chromatography instrument.

2.2.6. **Hypersensitivity Assay.** Isolate that was grown in nutrient broth (NB) (cell density ±10⁸ cell/mL) was injected on the below surface of tobacco leaf by using syringe (without needle) [25]. *Pseudomonas syringae* was used as positive control whereas *Bacillus cereus* and sterile media were used as negative control. Hypersensitivity respond was observed after 18 hours of injection time [26].

2.2.7. **Identification Based on 16S rRNA gene.** The DNA of isolate GPC3.7 was isolated following the Presto™ Mini gDNA Bacteria Kit protocol. The 16S rRNA gene was amplified by Polymerase Chain Reaction (PCR) method, using specific primer for bacteria, 63f (5’-CAGGCCCTAACACATGCAAGTC-3’) and 1387r (5’-GGGCGGWGT-GTACAAGGC-3’) [27]. 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 ddH₂O. PCR was done following condition: predenaturation (94 °C, 5 min), denaturation (92 °C, 30 min), annealing (55 °C, 30 sec), elongation (72 °C, 1 min), and postelongation (72 °C, 5 min), 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 by using Basic Local Alignment Search Tool-Nucleotide (BLASTN) from National Center for Biotechnology Information (NCBI). Phylogenetic tree were made by MEGA6 software [28].

2.2.8. **Growth Curve Observation.** One loop of bacteria isolate GPC3.7 was inoculated into 20 mL NB, incubated in shaker incubator until the cell density reached 10⁸ CFU/mL. One mL of culture was inoculated to 100 mL NB, incubated in shaker incubator (120 rpm, 30 °C) from 0 – 48 h. Cell density was measured every 3 h by using spectrophotometer Genesys 20 at 620 nm wavelength. Cell amount was determined by comparing with log cell curve upon cell turbidity of isolate GPC3.7.

### 3. Result and Discussion

3.1. **Result**

There were 44 phosphate solubilizing bacteria (PSB) isolates found from 15 soil samples around limestone mining area, Blindis mountain, Cirebon. Preliminary screening was done by observing the clear zone on Pikovskaya media (Figure 1a). The solubility index of all isolate was measured about 0.125 to 2.375. There were 22 PSB isolates were grown on N-free bromothymol blue (NfB) medium and 19 isolates were grown on Congo Red Agar (CRA) medium. Colonies that grew on NfB medium showed that the bacteria could fix free nitrogen because media did not content nitrogen source. Nitrogen fixing bacteria could change the color of NfB medium from green to blue (Figure 1b). Colonies that could absorb Congo red were indicated as nitrogen free fixing bacteria. Only 10 isolates were indicated as symbiotic living microorganisms whereas 12 others were categorized as N-free fixing bacteria.
There were 9 isolates that could not cause hypersensitivity symptoms (necrotic) on tobacco leaf after 48 h of incubation, compared to the hypersensitivity reaction caused by \( P. \) syringae as positive control, showed the forming of yellow-brownish spot on tobacco leaf. The description of 9 isolates are described in Table 1.

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

| No | Isolate code | Gram | P Solubility Index | Growth on media | Hypersensitivity to tobacco leaves |
|----|--------------|------|--------------------|-----------------|-----------------------------------|
| 1  | GPA2.1       | Negative | 0.304             | +++***          | +***                             |
| 2  | GPA2.2       | Negative | 0.263             | +              | ++***                            |
| 3  | GPC1.3       | Positive  | 0.444             | +++            | -                                |
| 4  | GPC1.7       | Positive  | 0.278             | +++**          | +++                              |
| 5  | GPC3.7       | Negative  | 2.375             | ++            | +++                              |
| 6  | GPC4.2       | Negative  | 0.714             | +              | +                                |
| 7  | GPC4.3       | Positive  | 0.206             | ++            | +++                              |
| 8  | GPC4.6       | Negative  | 0.250             | ++            | +++                              |
| 9  | GPC4.11      | Negative  | 0.229             | +             | +++                              |

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

Isolate GPC3.7 was chosen to be further observed, based on its P solubility index, N-fixing ability and growth stability. Phosphate quantitative estimation assay of isolate GPC3.7 was unmeasured (Figure 2). The isolate GPC3.7 did not form blue color complex after reacted with reagent, but yellow that could not be well read on 830 nm wavelength. Nitrogenase activity of isolate GPC3.7 was determined by the amount of ethylene concentration produced. The concentration of ethylene produced by isolate GPC3.7 was 0.291 ppm/h.
The colony of GPC3.7 morphologically had round shape, entire margin, raised elevation and white color. Based on Gram staining, isolate GPC3.7 was Gram negative bacteria with coccus cell shape. The phylogenetic tree showed that isolate GPC3.7 was closely relative to *Acinetobacter baumannii* with similarty level 100% (Figure 3).

The growth curve of isolate GPC3.7 were observed every three hours. The lag phase was not observed. Logarithmic phase was observed at 30 hours (11.793 CFU/mL). Stationer phase was observed from 33 – 36 hours (Figure 4).
3.2. Discussion

Plant growth promoting rhizobacteria (PGPR) is a group of bacteria that live and colonize around plant root or rhizosphere. Some PGPR are able to solubilize phosphate and fix nitrogen that beneficial for plant. Phosphate solubilizing and nitrogen fixing bacteria can be isolated from different kind of soils and habitats. Chen et al. [11] isolated PSB from subtropical soil in Taiwan. Bacteria that could fix N and are able to solubilize phosphate could be isolated from some species of lichens, namely Canoparmelia caroliniana, Canoparmelia crozalsiana, Canoparmelia texana, Parmotrema sancti-angelii and Parmotrema tinctorum [29]. Siddike et al. [30] isolated PSB and nitrogen fixing halotolerant bacteria from soil around coastal area. Phosphate solubilizing psychrotolerant bacteria from extreme region such as Antarctic had also been reported [31].

Phosphate solubilizing bacteria could be isolated by using Pikovskaya media. Phosphate solubilizing bacteria isolate that grow on Pikovskaya media generally form clear zone around the colony. Clear zone forming indicates the phosphate solubilization activity and acid production by bacteria [32]. Tricalcium phosphate (Ca$_3$PO$_4$) in media could be absorbed by plant in ion form (Pi, HPO$_4^{2-}$, H$_2$PO$_4^-$) [33].

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. A number of researches used NfB as selected media for nitrogen free living bacteria [34, 35]. CRA was used to distinguish nitrogen fixing bacteria that live freely and symbiotically with plant root. Congo red in the media could distinguish rhizobia group with nitrogen free living bacteria Azospirillum [19]. Congo red stains amyloid and bacteria cellulose, also interact with $\beta$-D-glucan and polysaccharide on its rhizobia group capsule [36]. YMA plus Congo red medium has the same function as CRA medium. Generally, YMA medium is used for cultivating nitrogen fixing symbiotic rhizobia such as Bradyrhizobium japonicum that could not absorb Congo red [37]. Selection process on NfB, CRA and YMA media could give us preliminary information about the ability of nitrogen fixation by bacteria. Isolate GPC3.7 could grow and change the color of NfB media from blue to green, as indication of pH changing. Isolate GPC3.7 also could not absorbs Congo Red that refer the isolate to root symbiotically bacteria.

Phosphate solubilization of isolate GPC3.7 was not quantitatively observed. Based on phosphate solubility curve (Figure 2), we could not define the correlation between cell growth (log cell) and the amount of soluble phosphate. The result implied that there was no correlation between qualitative and quantitative estimation, based on the observation of its growth on Pikovskaya agar and in Pikovskaya broth medium. Some researches had reported the same condition [12, 38]. The P soluble concentration of GPC3.7 might be lower than 1 mg/L.

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 $nif$L dan $nif$A), structural genes (such as $nif$H, $nif$D and $nif$K) and other supporting genes. The $nif$H gene regulates the Fe protein whereas $nif$D and $nif$K genes regulates Mo-Fe protein [39]. From preliminary screening, we assumed that isolate GPC3.7 qualitatively could fix nitrogen and live symbiotically. Nitrogenase activity was measured by

![Figure 4. Growth curve of isolate GPC3.7](image)
Acetylene Reduction Assay (ARA). Acetylene (C\(_2\)H\(_2\)) 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\(_2\)H\(_4\)) production [37]. The concentration of ethylene produced by isolate GPC3.7 was 0.291 ppm/h. Its activity was higher than bacteria that isolated from soil around Jambi forest, *Beijerinckia fluminensis* (0.094 ppm/h) [24].

The phylogenetic tree showed that isolate GPC3.7 was closely related to *A. baumannii* with similarity level 100% (Figure 3). Acinetobacter genus was known as strong phosphate solubilizer, but the name *A. baumannii* was not found as biofertilizer in any paper. Liba *et al.* [29] stated that chemoorganotrophic bacteria that could fix N from genus *Acinetobacter* could be isolated from lichen. Isolate *Acinetobacter* strain CR 1.8 from rice rhizospheric soil in Northern Thailand had been reported was able to grow on up to 25% NaCl, between 25 and 55 °C, and pH 5–9. The isolate also had protease activity [40].

4. Conclusion

Phosphate solubilizing bacteria that could fix N could be found from soil around limestone mining area in Cirebon. Isolate GPC3.7 had the highest solubility index (2.375), however its quantitative estimation could not be determined because the concentration of soluble phosphate might lower than 1 mg/L. Based on nitrogenase reduction assay, isolate GPC3.7 could convert acetylene to ethylene as much as 0.291 ppm/h. Isolate GPC3.7 was Gram positive bacteria. Identification based on 16S rRNA showed that isolate GPC3.7 was closely related to *Acinetobacter baumannii*.

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