Molecular Identification and Phylogenetic Analysis of Phosphate Solubilizing Bacteria *Aneurinibacillus migulanus* From Rhizosphere *Imperata Cylindrica*, Karst Citatah, Bandung Barat, Jawa Barat, Indonesia

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Abstract. Phosphate converted from insoluble phosphate bonds (through secretion of organic acids) to P-inorganic bonds by phosphate solubilizing bacteria (PSB). Two highly potential PSB isolates, BR.5 and BR.7, were isolated from rhizosphere *Imperata cylindrica*, ex-mining limestone land in Karst Citatah, Province Jawa Barat, Indonesia. From the screening of P-solubilizing test results, BR.5 showed the highest clear zones in pikovskaya agar containing Ca₃(PO₄)₂. The molecular methods to determine the species of bacteria are DNA extraction, DNA amplification by PCR, qualitative DNA testing by electrophoresis, and sequencing. The result of PCR results of DNA bands by electrophoresis, BR.5 has 1500 bp fragment length, 16S rRNA gene yielded on the phylogenic tree of Isolate BR. 5 has a similarity or relationship with the species *Aneurinibacillus migulanus* Isolate Am25 with a bootstrap value of 99%. Based on the genetic distance between Isolate BR.5 and *Aneurinibacillus migulanus*, Isolate Am25 has a distance value of 0.71%.

1. Introduction
The plant can uptake phosphate (P) as a macronutrient for plant growth, development, and metabolism[1]. Phosphate is an essential material to synthesize phospholipids, nucleic acids, ATP, and plays a role in regulating enzyme and metabolic pathways [2]. Soil accumulate P for roughly 0.04-0.1% (w/w). However, the plant only directly assimilated for a small fraction of P (H2PO4) [3]. Approximately, P-inorganic in the soil exists in an insoluble form, for example, Ca₃(PO₄)₂; and P-organic in the insoluble/soluble form (for example, phytic and nucleic acids) [4].

The location of the Karst area is in Kabupaten Bandung Barat, Jawa Barat, Indonesia. The extent is 10,320 ha consisting of 1,794 ha of rice fields and 8,526 ha of land with an altitude between 700-900 masl. The Citatah karst area is one of the oldest karst areas in Java, formed 20-30 million years ago [5]. Limestone mining activity induce damage to the physical condition of the soil. The destruction, such as there is bulk density (compaction), lack of essential nutrients, low pH, contamination of heavy metals in ex-mining areas, and decreased population of soil microbial activity. The low pH condition results in macronutrients added through fertilization to be ineffective because the nutrient will bound by metals [6].

In ex-mining land restoration activities, modifications can be made to trigger the succession stage (nudation, migration, ecesis, reaction, stabilization, and climax). This PSB from the rhizosphere is an
alternative technology to increase plant growth productivity on less fertile lands, such as the ex-mining area. Rhizobacterial isolated from the rhizosphere of rice from 4 districts in Southeast Sulawesi could dissolve phosphate and fix nitrogen [7].

The technique to identified PSB rhizobacteria is molecular method with PCR. Welsh and McCleland first used the PCR technique in 1990 as a genetic marker. According to [8], PCR or polymerase chain reaction is a specific DNA propagation technique in vitro by carrying out lengthening nucleotides from the primer simultaneously.

The four main components in the PCR process are (1) DNA prints, namely DNA fragments to be multiplied, (2) primary oligonucleotides, which are a short oligonucleotide term (15-25 nucleotide bases) which are used to initiate DNA chain synthesis, (3) deoxyribonucleotides trifopat (dNTP) consists of dATP, dCTP, dGTP, dTTP. (4) DNA polymerase enzyme, which is an enzyme that performs catalyzed reactions of synthetic DNA chains. Another essential component is the buffer compound [9].

To resolve the purpose of sustainable land improvement, the application of indigenous phosphate solubilizing bacteria provides a new approach to enhance the quality of karst soil. Indigenous microorganisms are more potential and sustainable than introducing microorganisms when applied as biological fertilizers [10]. Based on the introduction, we research Molecular Identification and Phylogenetic Analysis of Phosphate Solubilizing Bacteria *Aneurinibacillus migulanus* from Rhizosphere *Imperata cylindrica*, Karst Citatah, Bandung Barat, Jawa Barat, Indonesia.

2. Methods

2.1. Isolation and screening of phosphate solubilizing bacteria

Soil samples were taken randomly at three different points in the rhizosphere of the *Imperata cylindrica* plant that grows in the Karst Citatah, West Bandung Regency, West Java. Ten grams of the soil sample was put into a bottle with sterile aquadest. The soil sample was diluted to $10^{-7}$ dilution. From each series of diluted solution, 0.1 ml solution was poured on a Petri dish containing Pikovskaya Agar Media [11]. The most potential bacteria in P-solubilizing based on a clear zone score in Pikovskaya media will be continued to be identified in molecular methods.

2.2. DNA Extraction and 16S rDNA Sequence Analysis

The DNA extraction kit was using the Genomic DNA Presto ™ Mini gDNA Bacteria Kit (Geneaid). The procedure was carried out in 5 steps according to the Genomic DNA Presto ™ Mini Bacteria Kit protocol. DNA amplification by PCR method, PCR master mix preparation. All reagents are liquid and properly mixed. Prepare the PCR master mix with the right volume for all components. Calculate the volume required for each component based on the calculation (Kapa Biosystems, 2014). Preparation Using PCR Add Sample and PCR Master Mix according to the concentration count, and Templates and primers to the PCR tube or well from the PCR Plate. Amplification of 16S rDNA of Bacterial Isolates. Fragments of the 16S rRNA genes of each bacterial isolate were separately amplified using universal primers Forward Primer Bact 27F (5'- AGA GTT TGA TCM TGG CTC AG -3 '), Reverse Primer Uni 1492R (5'- TAC GGY TAC CTT GTT ACG ACT T -3 '). PC master mix (25 µl) contained 1µl of the extracted DNA, contained 1µl of the extracted DNA, 5 µl dNTPs, 1 µl of each of the primers 27F and 1492R, 1 µl of 5U / µl KAPA2G Taq Polymerase, and 5 µl PCR buffer. To this content reverse osmosis purified water up to a volume of 25 µl was added. The temperature Cycle on PCR Machines, Initial denaturation 95°C, 5 minutes (1 cycle), denaturation 94°C, 15 second, Annealing 52°C, 30 second, extension 72°C, 1 minute for 35 cycles, final extension, 72°C, 3 minutes, 1 cycles.

2.3. Electrophoresis

After the PCR process, the sample continued to electrophoresis by using TEB Gel 1% agarose. The result of electrophoresis was documented on gel documentation to observe the DNA bands.
2.4. Sequencing Results Nucleotide Analysis
The sequencing results were raw data taken from the 1st BASE Malaysia sequencing service company, processed using the BioEdit application and analyzed using the Mega Application (Molecular evolutionary genetics analysis) version 6.0 [12].

3. Result and Discussions

3.1. Isolation and screening of phosphate solubilizing bacteria
From 15 samples collected and processed, a total of five isolates of bacterial were purified. From five isolates of bacteria, 2 bacteria formed the clear zone. The PSB selective medium for isolation is Pikovskaya agar. PSB that grows on this media will form colonies around clear areas (clear zones) [13]. The table shows that BR.5 has the highest average in P-solubilizing ability. After the screening result, the best isolate continued to molecular identification.

| Isolate | Average clear zone (mm) |
|---------|-------------------------|
| BR.2    | -                       |
| BR.3    | -                       |
| BR.4    | -                       |
| BR.5    | 65                      |
| BR.7    | 3                       |

3.2. DNA Extraction and Qualitative test of DNA visualization
The DNA of the BR.5 sample was extracted by Genomic DNA Presto™ Mini gDNA Bacteria Kit (Geneaid). The quality of DNA was tested in TEB Gel 1% agarose by comparing the DNA band's sharpness with the marker [14]. The visualization of the BR.5 sample in figure 1, showed a band located at 1500 bp. Claridge [15] demonstrate that nucleotides with a size between 1500-1600 nitrogen bases were included bacteria. The DNA extraction process was sent to 1st BASE Malaysia to continue in the sequencing process.

![Figure 1](image.jpg)

**Figure 1.** The results of the Visualization of the 16s rRNA Gene of Bacteria Isolate BR.5.

3.3. Nucleotide Analysis of DNA Sequencing
The sequencing results of the BR.5 were processed using BioEdit software and analyzed using Mega 6.0. The result was in the electropherogram graph, there were differences of color in the type of nucleotide base, A (Adenine) is green, G (Guanine) is black, C (Citosin) is blue, and T (Thymine) is
red. From the electrophoregram graph, the sequencing result BR.5 with 27F forward primer was 728 nitrogen-base sequences, and 1492R reverse primers were 742 nitrogen-base sequences. The electrophoregram graph's results with both forward 27F and 1492R reverse primers show that the quality of the DNA was not sufficient because there was an N or Noise sign on the graph. The N sign indicates the sequencer machine cannot read the nucleotide sequence correctly due to the accumulation of nucleotide sequences on the electrophoregram graph. The excellent quality of nucleotides sequence without N (Noise) produce by replaced the unreadable nitrogen base sequence manually by determining the highest peak on the electrophoregramer according to its color. The excellent quality nucleotide from total DNA isolates BR.5 from continuous to analyze by Mega version 6.0[16]. The result is described in Figure 2:

Figure 2. BR.5 Isolate Nucleotide Base Sequences

3.4. Phylogenetic Tree Reconstruction of BR.5 Bacterial DNA Isolates

Phylogenetic tree analysis is a technique to determine the origin and history of species[17]. The method to develop a Phylogenetic tree is by comparing the nucleotide sequence of isolate BR.5 with the nucleotide sequence of other species in NCBI (Gene Bank) and analyzed the result by maximum likelihood method in the Mega application (Molecular evolutionary genetics analysis) version 6.0 [18]. This phylogenetic tree analysis aims to precisely construct the relationships between organisms and estimate the differences between ancestors and offspring [19]. The phylogenetic tree is described in figure 3:
Based on Figure 3, BR.5 isolate belongs to *Aneurinibacillus migulanus* isolate Am25 species with a bootstrap value of 99%. Bootstrapping is a method to confirm the group's stability on phylogenetic trees and branches [20]. The bootstrap value among isolate BR.5 and *Aneurinibacillus migulanus* Am25 was 99%. This indicates the stability and reliability of the phylogenetic tree were high. According to Efron et al. [20], the bootstrap confidence level is above 95% categorize as stable, and below 70% means unstable. *Aneurinibacillus migulanus* or *Bacillus brevis* has a rod shape, produce endospores, aerobic or facultatively anaerobic, and gram-positive bacteria. These bacteria live in various environmental conditions. The spores are heat resistant, cold, radiation, desiccation, and disinfectants. As Bacillus' physiological characteristics and their ability to produce enzymes, antibiotics, and other metabolites, they can be used in various medical, pharmaceutical, agricultural, and industrial processes. The example of antibiotics produced by Bacillus is Bacitracin and polymyxin [22].

4. Conclusion
The screening test in pikovskaya agar media, the isolate BR.5 showed, has P-solubilizing ability. BR.5 had a fragment length of 1500 bp 16S rRNA gene from molecular identification, BR.5 isolate belongs to *Aneurinibacillus migulanus* isolate Am25 species with a bootstrap value of 99%.
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