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IDENTIFICATION OF POTENTIAL ISOLATE PHOSPHATE SOLUBILIZING BACTERIA USED 16S rRNA GENE

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ABSTRACT: *Plants always interact with microorganisms either negatively or positively during their life cycle. The Phosphate Solubilizing Bacteria (PSB) group is one example that has some beneficial effects on the plant. PSB can transform insoluble soil inorganic phosphates into soluble phosphorous so plants can absorb them. This research aimed to test the isolate's ability to solubilize phosphorous and use the 16S rRNA marker gene to identify it. Testing the ability of bacteria to solubilize phosphorous on Pikovskaya's agar medium used a qualitative method by observing the halo zone in the colony around it. Then the bacterial isolate was identified by analyzing the result of sequencing the 16S rRNA gene. The RZ02 bacterial isolate showed the highest performance in solubilizing phosphorous, with a 9.1 mm solubilization index. The gene of 16S rRNA was carried out by using primers 27F and 1492R with an amplicon size of 1500 base pairs. Sequencing analysis and construction of a phylogeny tree were conducted by the Mega X neighbor-joining method and showed 100% similarity with Pseudomonas aeruginosa OIS 481. The solubilization index indicated that the bacteria had a potential candidate to be one of the inoculant components for biofertilizer.*

Keywords: *Phosphate Solubilizing Bacteria, Biofertilizer, Pseudomonas aeruginosa, 16S rRNA.*

INTRODUCTION

Microorganisms are extremely diverse and play an important part in many sectors of life, including health, industry, and agriculture. They have a significant impact on plant growth, particularly in terms of enhancing productivity and health, because they can alter the root environment, either stimulating or inhibiting plant development (Gondal *et al.*, 2021). Furthermore, they naturally have the ability to maintain soil fertility because they provide nutrients in an available form for plants.

Microorganisms that can help in the process of plant growth are located in the soil and the rhizosphere. Moreover, they allow them to form epiphytic or endophytic associations in plant tissue. Because of this ability, they have been known as plant growth-promoting microorganisms. Regarding their effect on plants, these microorganisms have been grouped as biostimulants, biocontrol, biofertilizers, and bioremediation (Gangwar *et al.*, 2017; Kumar *et al.*, 2016).

The uncontrolled use of chemical fertilizers and pesticides has reduced land fertility over time. It gets special attention regarding environmental deterioration and low production in agriculture. Furthermore, with the motto

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"back to nature" gaining popularity in society, farmers are looking for a low-cost and environmentally friendly alternative to chemical fertilizer (Kumar *et al.*, 2022; Tomer *et al.*, 2016). In terms of the context, biofertilizer emerges as an essential component for sustaining healthy land because it consists of different types of microbes that live in the rhizosphere. They can promote plant growth by fixing nitrogen from the air, transforming macronutrients into micronutrients, and solubilizing phosphate, zinc, and potassium into available forms (Don & Diep, 2014; Mishra *et al.*, 2013). In addition, the microbe inoculant is used as a biocontrol agent by producing an antagonist compound and interrupting pathogen quorum sensing. Therefore, microbes have several ways to enhance plant growth (Suyal *et al.*, 2014).

Phosphate Solubilizing Microorganisms (PSM) are one of the components of biofertilizers. Bacteria, fungi, mycorrhizae, actinomycetes, and algae dissolve and mineralize phosphate (Sharma *et al.*, 2013). Several soil bacteria, including *Pseudomonas* spp., *Bacillus*, and *Burkholderia*, have been shown to be capable of dissolving phosphate into a usable form for plants via solubility and mineralization (Babalola & Glick, 2012; Istina *et al.*, 2015; Raj *et al.*, 2014). Phosphate Solubilizing Bacteria (PSB) can make P available to plants by three mechanisms: 1) pH drop, 2) chelation, and 3) mineralization. Organic acids are secreted to lower the pH; organic acids that have carboxyl and hydroxyl groups will chelate cations from phosphate, which bind to Al, Ca, Fe, and Mg so that they are accessible forms.

Immobilized organic phosphates are mineralized from the remains of microorganisms, plants, and animals that contain much phosphate in the form of nucleic acids, phosphate sugars, phospholipids, and phytic acid in soil. The phosphate mineralization and immobilization process are critical in the natural P cycle (Kalayu, 2019; Sharma *et al.*, 2013).

This study aimed to identify the species of phosphate-solubilizing bacteria using the 16S rRNA marker gene, which produced more accurate findings than phenotyping. As it is ubiquitous, practical, and reliable, the utilization of the 16S rRNA gene as a molecular marker is frequently the first choice. This gene's nitrogen base sequence region is both conservative and hypervariable. Because of the slow evolutionary changes, the conservative portion is used to know the link between bacterial species by constructing a phylogenetic tree. The various sections determine the diversity of strains in one species (Akihary & Kolondam, 2020; Hassler *et al.*, 2022). Based on in vitro tests (Sembiring & Sumanto, 2019), the selected isolates indicate antifungal activity against *Fusarium oxysporum* f.sp *cubense*, but have not yet been evaluated for their ability to solubilize phosphate. As a result, it is interesting to investigate these bacteria to promote possible isolate candidates as biofertilizers.

METHODS

The materials used were three bacterial isolates, King's B agar and liquid media, Pikovskaya agar media, Agarose 0.8%, 1x TBE buffer, Presto mini gDNA Bacteria Kit GBB100 (Geneaid), PCR MyTaqHS Red Mix (Bioline) A pair of

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primers 27F: 5'AGAGTTTGATCMTGGCTCAG-'3 and 1492 R: 3'-TACGGYTACCTTGTTACGACTT-'5, DNA ladder 1000 bp. The types of equipment are petri dishes, analytical balance, incubator, PCR machine, shaker, electrophoresis, nanodrop, micropipette, sequencer (AB 3130 Genetic Analyzer), PCR visualization device and Mega X.

Phosphate Soulubilizing Test

The isolates were cultured for 24 hours at room temperature on King's B agar. The ability of bacterial isolates to solubilize phosphate was carried out qualitatively by spotting one loop of bacterial colonies in three quadrants of Pikovskaya agar media, which were incubated at room temperature for 7 days. The presence of a clear zone formed around the colony indicated that the bacterial isolate was able to solubilize phosphate. The isolate had the highest dissolution index, which would be identified using the 16S rRNA gene. The phosphate solubility index was calculated using the following formula.

$$\textbf{Clear Index} = \frac{\textbf{Clear Zone Diameter} - \textbf{Colony Diameter}}{\textbf{Colony Diameter}}$$

Isolation of Genome DNA

Selected bacterial isolates were cultured in King's B liquid for 24 hours at room temperature in a shaking incubator at 150 rpm. Afterwards, the genomic DNA was extracted according to the Presto mini gDNA Bacteria (Geneaid/GBB100) Kit Presto protocol. Pellets were obtained by removing the supernatant after centrifuging 1 ml of bacterial culture in an Eppendorf tube for 60 seconds at 15,000g. Subsequently, 180 µl of GT buffer and 20 µl of proteinase K were added. The mixture solution was resuspended with a micropipette and transferred to the GD column after being mixed with 200 µl of GB buffer and 200 µl of absolute ethanol. Genomic DNA was obtained by washing and eluting of the GD column using a washing and elution buffer.

Amplification of the 16S rRNA Gene

One microlitre of the obtained genomic DNA sample (template) was put into a PCR test tube with a mixture of 12.5 µl of MyTaq Red Mix, each 0.5 L of forward (27F:5'-AGAGTTTGATCMTGGCTCAG-'3) and reverse primers (1492R: 3'-TACGGYTACCTTGTTACGACTT-'5), 10.5 µl ddH2O. Then, the reaction solution was placed into a PCR machine for amplification under the following conditions; initial denaturation for 1 minute at a temperature of 95^{o}C; denaturation of 95^{o}C for 15 seconds; annealing of 52^{o}C for 30 seconds; and extension of 72^{o}C for 45 seconds, which was repeated for 35 cycles. The amplification results were then verified on a 0.8% agarose gel with 100 volts for 40 minutes. The agarose gel was soaked for 15 minutes in EtBr (ethidium bromide) and distilled water solution, respectively. The agarose gel was viewed on a UV transilluminator to see the amplification. Positive results were obtained when a ± 1400 bp band was present. If the findings indicate a clean band without polish, the PCR band can be purified for sequencing using the zymo research kit.

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The 16S rRNA Gene's Nucleotide Sequence Analysis

The purified PCR products were sequenced using the Big Dye Terminator kit using the dideoxy sanger principle and the bidirectional PCR method. The sequencing data were analyzed with bio-edit and chromas pro software before being compared to gene bank data on the website www.ncbi.nlm.nih.gov using the BlastN tool. The ancestry comparison results from the database can be depicted as a phylogenetic tree using the Mega X software's neighbor-joining method.

RESULT AND DISCUSSION

According to qualitative tests on the three selected test isolates, the bacteria present varied phosphate solubilizing abilities, defined by clear zones around the colony on the Pikovskaya medium. The dissolution index (IP) check revealed that the RZ02 isolate had the maximum ability to dissolve phosphate compared to the other two isolates, with 9.1 mm for seven days of incubation (Figure 1A). The clear zone expands when phosphate minerals not dissolved in the media (calcium phosphate or hydroxyapatite) get dissolved because of biological chemicals produced by bacteria.

Regarding that, the clear zone formed determines the ability of microorganisms to dissolve phosphate, which can be done in three ways: secreting organic acids or exopolysaccharides (biochemical mineralization) or phosphatase enzymes (biological mineralization) (Heuck *et al.*, 2015; Osińska-Jaroszuk *et al.*, 2015; Sharma *et al.*, 2013). The concept of bacteria producing organic acid compounds is the most widely accepted and reported of the various mechanisms in phosphate solubilizing (Khan *et al.*, 2014). The amount and type of organic acid released are essential in phosphate dissolution, although the quality of the organic acid secreted is more critical than the quantity (Monica *et al.*, 2015).

Measuring on RZ02 revealed that the bacteria diameter did not considerably expand from day 4 to day 7. The clear zone that formed dropped and subsequently rose until the end of the measurement (Figure 1B). The interesting finding is that the clear zone was clearer, indicating that the phosphate solubilizing process may still be on, although the clear zone index has not increased.

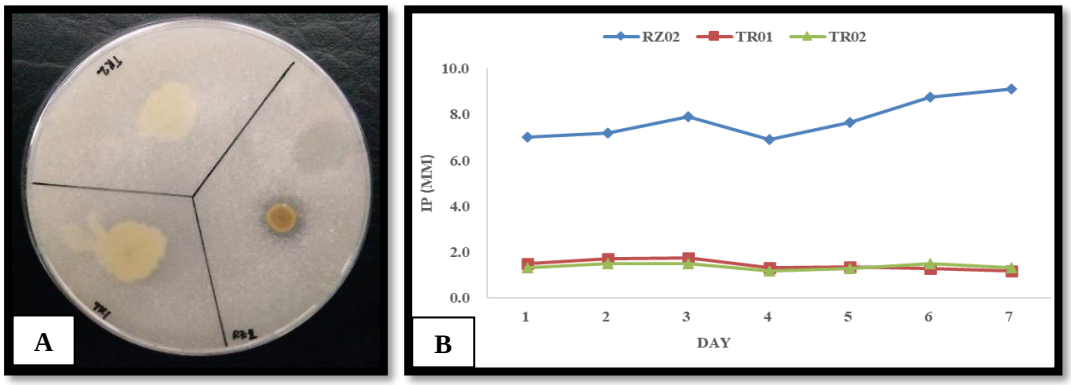

Figure 1. A) Phosphate Dissolution Clear Zone; dan B) Daily Phosphate Dissolution Index.

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After determining that RZ02 is a potential bacterium for biofertilizer inoculant, identification was performed through the 16S rRNA gene. The identification procedure was started by isolating the bacterial genome in the following stages: sample preparation, extraction, lysis, washing, and elution. Following the Presto Mini Kit Geneaid procedure, the isolated genomic DNA was utilized as a template in a PCR process to amplify the 16S rRNA gene, producing a single band of roughly 1500 base pairs (bp) (Figure 2). The 16S rRNA gene sequencing was aligned using the Nucleotide Blast tool on the GenBank ncbi.nih.nlm.gov to get the RZ02 species. The result showed that the isolate was 100% similar to *Pseudomonas aeruginosa*.

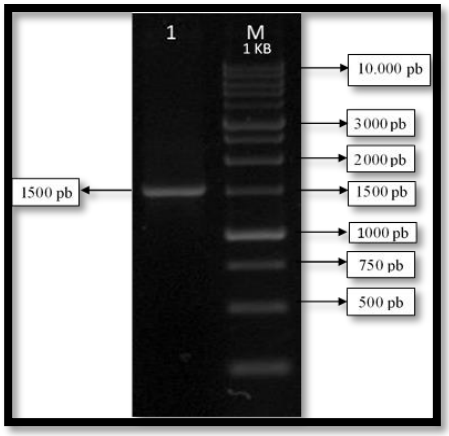

Figure 2. 16S rRNA Gene Amplification. 1) Isolate RZ02 (M) Marker 1 Kb.

Identification with the 16S rRNA marker gene is a ribosomal-based molecular technique frequently used because the 5S rRNA molecule is relatively small and not typical for analysis. In comparison, the 23S rRNA molecule has a secondary and tertiary structure that makes analysis challenging. According to (Akihary & Kolondam, 2020; Inderbitzin *et al.*, 2020), 16S rRNA molecules are advantageous when used as marker genes because they are almost present in every bacterium in the gene group or operon system. They have a conservative and varied region with a length of 1500 bp, representing informational purposes.

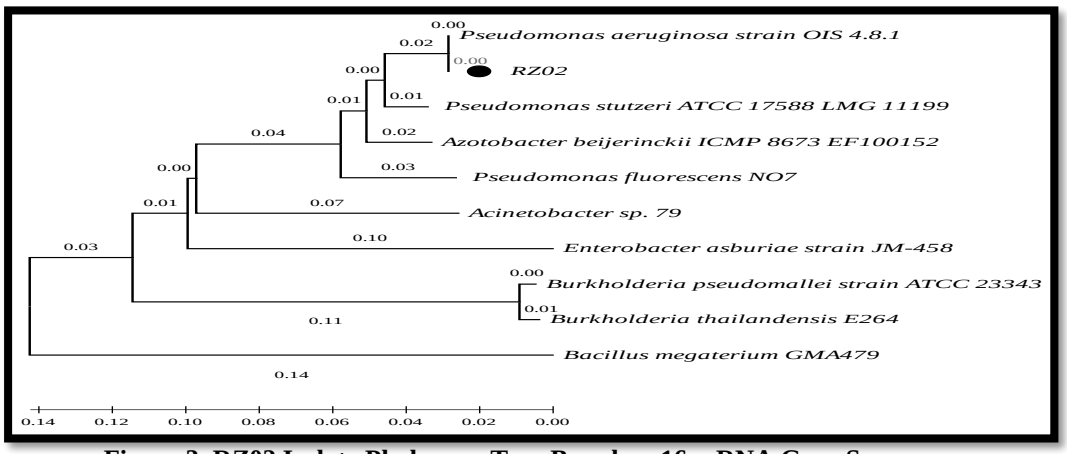

Figure 3. RZ02 Isolate Phylogeny Tree Based on 16s rRNA Gene Sequence.

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The construction of a phylogeny tree using the neighbor-joining method on Mega X software is depicted in Figure 3. The method constructs a phylogenetic tree from evolutionary distance data. This method is claimed to be better for creating phylogenetic trees since it has the principle of finding pairs of operational taxonomic units (neighbors) to minimize the number of variations in each cluster of taxonomic operational unit pairs (Abdelaziz & Assia, 2020). The phylogenetic tree revealed that the RZ02 bacteria matched similarities with *Pseudomonas aeruginosa* because they were on the same branch.

Pseudomonas bacteria are rod-shaped, gram-negative, polar-flagellated bacteria that have been identified as soil bacteria that induce positive interactions by growing plants optimally. The beneficial effects comprise a biocontrol agent, a growth regulator producer, or a biofertilizer. According to (Suresh *et al.*, 2022), *Pseudomonas aeruginosa* VSMKU3054 showed strong antibiosis potential against bacterial wilt caused by *Ralstonia solanacearum* in tomato by generating 2,4-diacetylphloroglucinol (DAPG) molecules. Besides, it germinated faster than the untreated controlbecause *Pseudomonas aeruginosa* could synthesis IAA. The same result was also conveyed by (Durairaj *et al.*, 2017) that in vitro experiments of *Pseudomonas aeruginosa* D4 could solubilize phosphate and testing on tomatoes could induce defense mechanisms. Another finding (Linu *et al.*, 2019) claimed that *Pseudomonas aeruginosa* PS2 and PS3 could produce IAA and dissolve phosphate. As a result, they increased the yield and weight of chilli in the greenhouse.

CONCLUSION

Three bacterial isolates tested in this study had the ability to solubilize phosphate qualitatively. RZ02 bacteria, which has the highest activity in solubilizing phosphate, is a potential bacterium as a biofertilizer inoculant and has been identified through a molecular approach using the 16S rRNA gene. The findings of nucleotide blasts in the GenBank database and phylogenetic tree construction revealed that the strain was identical to *Pseudomonas aeruginosa* OIS 481.

RECOMMENDATION

The information on the phosphate solubility index obtained from this research was limited to a qualitative test based on halo zone observation. Getting more accurate data on the orthophosphate produced can be quantified by employing the spectrometric or chromatographic method as a quantitative test.

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