Potential phosphate solubilizing bacteria from the rhizosphere of *Cotylelobium melanoxylon* tree

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**Abstract.** Microorganism have important roles in soil cycles and plant living activities. Phosphate solubilizing bacteria is one of the microorganisms in the soil that plays a role in helping provide phosphorus nutrients that are not available to plants. The purpose of this study was to obtain potential phosphate solubilizing bacteria from the rhizosphere *Cotylelobium melanoxylon* plants. Soil samples were taken in a composite at a depth of 0-20 cm from the rhizosphere area of *C. melanoxylon* plants in the village of Bona Lumban, Tukka District, Tapanuli Tengah Regency, North Sumatra. Isolation of phosphate solubilizing bacteria using the dilution plate technique with Pikovskaya medium. Phosphate solubilizing bacterial isolates were selected qualitatively by measuring the ratio of the clear zone. Selected isolates are identified up to species molecularly. The isolation results obtained were 16 isolates of phosphate solubilizing bacteria with a clear zone ratio varying between 1.03 to 1.68. Four isolates were selected with the highest clear zone ratio. Based on molecular identification, the four isolates included *Pseudomonas aeruginosa* species.

1. **Introduction**

Rhizosphere is an area around plant roots which is biologically and chemically still influenced by plant roots [1,2]. Rhizosphere is an area with high microorganism activity, due to various rhizosphere microbial inhabitants such as fungi, bacteria and actinomycetes that live and move around plant roots [2,3]. Rhizosphere is strongly influenced by plant metabolism through the secretion of various compounds from root exudates such as organic acids, amino acids, growth regulators, sugars, and vitamins. Rhizosphere is a very dynamic habitat that is different from soil that at is not vegetated. These differences are mainly related to the presence of microbial communities [1,3].

Various microbes that live around the roots have their respective functions and roles so that their functions and roles affect plant growth. One of them is phosphate solubilizing microbes that help increase the availability of phosphorus nutrient. Phosphorus nutrient is needed by plants and microbes that function in the process of plant metabolism by supplying energy for the metabolic process. In the soil, the phosphorus nutrient is bound to soil component so that its availability is low. High solubility of aluminum and iron in acid soils, and high solubility of calcium in alkaline soils causes the formation of phosphorus compounds which are insoluble [4-8]. Low availability of phosphorus is one of the factors limiting plant growth, so phosphate fertilizer is used in excess of the dose to get maximum results. Phosphate solubilizing microbes have the ability to release these bound P and become available to plants. Phosphate solubilizing microbes consist of bacteria, fungi and a little actinomycetes. Genus *Pseudomonas, Bacillus, Enterobacter, Burkholderia, Flavobacterium,*
Rhizobium, Arthrobacter and Serratia is a genus of bacteria in the soil that acts phosphate solubilizing bacteria. The genus Pseudomonas and Bacillus are the genus with the greatest ability to dissolve phosphate [3,6,8].

Cotylelobium melanoxylon is one of the plants in North Sumatra which is used by the local community as a medicinal plant and enhancing the aroma of traditional alcoholic drinks [9,10]. Soil in rhizosphere has potential as a habitat for various types of microbes that are useful for increasing plant growth, so microbial exploration under C. melanoxylon stands is needed. The purpose of this study was to obtain potential isolates phosphate solubilizing bacteria from the rhizosphere of C. melanoxylon plants.

2. Materials and Method

2.1. Soil sampling
Soil sampling was carried out systematically at six points at a depth of 0-20 cm around the roots of Cotylelobium melanoxylon tree in Bona Lumban Village, Tukka District, Tapanuli Tengah Regency, North Sumatra. The soil sample is then compiled. Soil samples are put in plastic and placed in ice boxes for transportation from the field to the laboratory.

2.2. Isolation of phosphate solubilizing bacteria
Phosphate solubilizing bacteria were isolated using dilution plate technique with Pikovskaya medium containing insoluble tricalcium phosphate [11, 12]. The soil samples were diluted up to 10\(^{-5}\) dilution, plated on petridishes and incubated at 27-30 °C for five days. At the end of incubation, phosphate solubilizing bacteria colonies were visually identified from the clear zone around the bacterial colony. The colonies were sub cultured, purified and maintained in Pikovskaya’s agar slants.

2.3. Qualitative isolates selection
Qualitatively isolate selection using Pikovskaya solid medium. All purified isolates were grown on solid Pikovskaya media containing insoluble tricalcium phosphate. Isolates were incubated for five days, recorded colony diameter and clear zone diameter. Then the ratio of the clear zone is calculated by dividing the diameter of the colony with the diameter of the clear zone [13]. The potential isolates were isolates with the highest clear zone ratio.

2.4. Molecular identification of potential isolates
Potential isolates were also identified using molecular marker. ITS 1 rDNA primer (5’ TCC GTA GGT GAA CCT TGC GG 3’) and ITS 4 (5’TCC TCC GCT TAT TGA TAT GC 3’) according to [14] were used in this research. The molecular activities conducted through DNA isolation, PCR amplification, sequenching and sequence analysis.

3. Result and Discussion

3.1. Isolation and qualitative selection of phosphate solubilizing bacteria
The presence of phosphate solubilizing bacteria in the soil is influenced by the availability of nutrients and the content of organic matter. The higher availability of nutrients, the higher the bacterial population. Organic matter is the energy source and carbon source of most of the microbes that live in the soil, so the presence of microbe will follow the content of organic matter. So that, the high content of organic matter in the rhizosphere of C.melanoxylon plants has the potential to increase microbial population in the soil. Therefore the presence of litter on the forest floor maintains the survival of soil microbes. In this case including phosphate solubilizing bacteria. In the soil phosphate solubilizing microbes population range from 10\(^4\) to 10\(^6\) Colony Forming Unit (CFU) per gram of soils [15,16]. In this study, the population of phosphate solubilization bacteria obtain was 3.17x10\(^4\) CFU per gram of soils with 4.72% organic matter content.
Sixteen phosphat solubilizing bacteria from rhizosphere of *C. melanoxylon* were isolated. Phosphat solubilizing bacteria are characterized by clear zone formation around the colony (figure 1), which indicated the dissolution of phosphate insoluble by bacteria. Phosphat dissolution occurs because bacteria secrete organic acids in the metabolic process, so that the phosphate which initially bound to calcium becomes released.

![Clear zone](image)

**Figure 1.** Clear zone around the colony of phosphate solubilizing bacteria

Sixteen isolates were selected qualitatively by measuring the clear zone ratio (table 1). The ratio of obtained clear zone varies from 1.03 in BD8 to 1.64 in BD4. The higher value of clear zone ratio indicated greater ability for the bacteria to release the phosphate bound. Phosphate release is related to organic acid produced by each isolate, because each isolate produce different type and amount of organic acid [17].

| No. | Isolate code | Colony diameter (cm) | Clear zone diameter (cm) | Clear zone ratio |
|-----|--------------|----------------------|--------------------------|-----------------|
| 1   | BD1          | 0.80                 | 1.07                     | 1.33            |
| 2   | BD2          | 2.52                 | 2.78                     | 1.10            |
| 3   | BD3          | 2.85                 | 3.04                     | 1.06            |
| 4   | BD4          | 0.82                 | 1.38                     | 1.68            |
| 5   | BD5          | 1.03                 | 1.72                     | 1.67            |
| 6   | BD6          | 0.45                 | 0.75                     | 1.67            |
| 7   | BD7          | 2.38                 | 2.68                     | 1.13            |
| 8   | BD8          | 3.02                 | 3.12                     | 1.03            |
| 9   | BD9          | 0.99                 | 1.17                     | 1.18            |
| 10  | BD10         | 0.50                 | 0.60                     | 1.20            |
| 11  | BD11         | 1.00                 | 1.53                     | 1.53            |
| 12  | BD12         | 1.10                 | 1.20                     | 1.09            |
| 13  | BD13         | 0.75                 | 0.85                     | 1.13            |
| 14  | BD14         | 0.45                 | 0.55                     | 1.22            |
| 15  | BD15         | 0.90                 | 1.00                     | 1.11            |
| 16  | BD16         | 0.55                 | 0.65                     | 1.18            |

Various type of organic acid with low molecular weight are produced by phosphate solubilizing microbes from the result of metabolic process such as citric acid, oxalic acid, lactic acid, acetic acid and formic acid. Organic acid will form chelate with P binding component such as AL, Fe and Ca so that P become available. Phosphorus dissolution is associated with a decrease in media pH due to organic acid produced by microbes [17,18]. According to [19,20] ability of organic acids to release P bound was different, where the ability of citric acid was greater than oxalic acid, oxalic acid was as effective as tartaric acid and malic acid. Both of these acid have greater ability to release P compared
to lactic acid, acetic acid and formic acid. Based on clear zone ratio value, selected four isolates which have the highest value of the clear zone are BD4, BD5, BD6 and BD11. This isolates then molecularly identified.

3.2. Molecular identification of selected isolates
The ITS region of rDNA sequences is shown in Table 2. Sequence analysis of the ITS regions of nuclear-encoded rDNA showed a significant alignment of 100% with the previous isolated bacterial species. The results of the 5.8 S rDNA sequence alignment revealed one genera of Pseudomonas that is Pseudomonas aeruginosa.

| Bacterial isolate | Species identified | Length | Identity | Accession number |
|-------------------|--------------------|--------|----------|------------------|
| BD4               | Pseudomonas aeruginosa | 2521   | 100%     | MH001386.1       |
| BD5               | Pseudomonas aeruginosa | 2468   | 100%     | LR130537.1       |
| BD6               | Pseudomonas aeruginosa | 2532   | 100%     | LR130537.1       |
| BD11              | Pseudomonas aeruginosa | 2423   | 100%     | MK205174.1       |

Pseudomonas is a bacterium that has fairly wide distribution in nature, it can be found in various habitats with good adaptability. P. aeruginosa, a proteobacteria is a member of the Pseudomonadaceae family. This bacteria can be isolated from various materials such as soil, water, plants, animals and humans. Uniquely, P. aeruginosa can thrive and survive in a variety of temperature and infrequent nutrition [21]. The genus Pseudomonas is a bacterial genus other than Bacillus which has a high ability to release insoluble P. Several Pseudomonas species have been reported the most efficient phosphate solubilizing bacteria, secretion of plant growth regulators and suppression of soil borne pathogens [22]. Pseudomonas aeruginosa which is isolated from the soil in addition to functioning as a phosphate solubilizing bacteria which capable solubilized phosphate significantly [23,24] also plays a role as plant growth promoting rhizobacteria (PGPR) [24]. Other than that P. aeruginosa is a microbial mediated biotic stress tolerance in plants, biological control microbes, produced indole acetic acid (IAA) and siderophores [23-26]. Better phytoremediation potential of broad bean plants grown in oily sand, significant enhanced in yield and uptake of nitrogen and phosphorus by grains and increase plant growth in spinach [3].

4. Conclusions
Sixteen phosphate solubilizing bacteria were sucessfully isolated from C. melanoxylon rhizoshere. Based on the clear zone value, four isolates were selected those where BD4, BD5, BD6 and BD11. Molecular identification showed that all of isolates were Pseudomonas aeruginosa.

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