Antibacterial and phosphate solubilization activity of endophytic bacteria isolated from Pteridophyta (*Tectaria barberi*)

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Abstract. Endophytic bacteria have been isolated from many plants and their contribution to host plants have been proposed by many researchers, such as preventing plant from pathogens, producing plant growth hormones, producing bioactive compounds, solubilizing phosphate, etc. However, the study of endophytes of pioneer plant such as fern is very limited. The objective of this study was to obtain potential endophytic bacteria from fern *Tectaria barberi* and to measure their ability to inhibit the growth of human pathogenic bacteria (*Escherichia coli* (EPEC), *Staphylococcus aureus* ATCC 29213, *Listeria monocytogenes* BTCC B693, Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300, *Staphylococcus epidermidis* ATCC 12228, and *Proteus vulgaris* ATCC 13315). The ability of isolates in solubilizing phosphate was measured to learn possible role of endophytes for the plant. Six endophytic bacteria were obtained. Antagonistic test indicated all isolates inhibited the growth of tested pathogens to some extends. Isolate EPS36 showed the highest inhibition activity against EPEC (with inhibition zone 8.6 mm). The isolate also inhibited the growth of *S. aureus* and *S. epidermidis* by 6.1 and 6.6 mm, respectively. EPS37 inhibited the growth of *S. aureus* by 6.1 mm, EPS02 inhibited *P. vulgaris* (5.6 mm), EPS42 to *L. monocytogenes* (5.7 mm), and EPS21 inhibited MRSA (5 mm). Qualitative screening of isolates in Pikovskaya media after 24 hours incubation showed that EPS41, EPS36, and EPS37 formed clear zone surrounding the colony indicating their ability to solubilize phosphates. As far literatures has been surveyed, this is the first study of endophytes bacterial isolates from pteridophytes in the country, and a lot more is needed in order to elaborate the role of endophytes from this plant.

1. Introduction

As a mega-biodiversity country, Indonesia has a large amount of species diversity, including ferns (Pteridophytes). The total number of ferns around the world is about 10,000 species, and most of them grow in Indonesia [1]. *Tectaria barberi* is one of fern species from the family of tectariaceae often found in tropical area, especially Indonesia. Tectaria genus grows in terrestrial area of tropical rainforest [2]. In Sumatra, tectaria particularly can be found in many regions, including in Sibolangit forest [3]. Ferns are highly beneficial to human life; it can be used as organic fertilizer, medicinal plant, as well as it possess ecological function to prevent erosion in forest ecosystem [4].

However, a comprehensive analysis of the role of ferns particularly on the view of associated microbes both in rhizospheric plant and inside of plant tissue or endophytes as well as their potential
has been very limited. Endophytes are endosymbiotic microorganisms which colonize plant tissues without causing any damage to the plants [5]. Naturally, endophyte functions as a great producer of bioactive compounds often used by plants to protect themselves against pathogen and often produce plant growth hormones [6, 7]. In addition, endophyte is often known to secrete multifunctional secondary metabolites; it can be the source of antiarthritic, antimicrobial, anticancer, antidiabetic, antiperspirant as well as having immunosuppressant activity [8,9].

Research on endophytic bacteria has been conducted on various types of plants, which mostly is medicinal plants such as Panax ginseng, Allamanda Cathartica Linn, and Curcuma longa L [10,11,12]. However, research on endophytic bacteria from the Pteridophyta of genus Tectaria has never been conducted. Here we reported the first study of endophytic bacteria from fern Tectaria barberi from the island of Sumatera. In addition, the endophytic bacteria were tested for their antibacterial activity and activity to solubilize phosphate in order to determine its future application prospect.

2. Materials and Methods

2.1. Isolation of Endophytic Bacteria from Tectaria barberi

Endophytic bacteria was initially isolated from T. barberi root. The isolation of endophytic bacteria from plant tissue begins with its surface sterilization process as reported by Hallmann et al. [13]. Roots were cleaned from dirt and soil particle and rinsed with running water. Afterwards, the root surface was brushed and then cut into small pieces of 1-2 cm. The sample was then soaked separately into ethanol 70% for 3 minutes and transferred into sterile distilled water. After that, the sample was placed into 1% sodium hypochlorite for 5 minutes and transferred in sterile distilled water, then in ethanol 70% for 1 minute. Samples were taken out from 70% alcohol and put into sterile distilled water for 2 minutes. Samples were placed on the surface of filter paper aseptically, and finally were placed on the surface of Nutrient Agar (NA) media supplemented with ketoconazole antibiotics (0.3 g/100 ml). The former cut was positioned towards the media. Samples were incubated at 37 ± 2°C for around 3 days. Appearing colonies were then transferred into new NA plate to obtain pure isolates for further analyses.

2.2. Morphology Characterization and Biochemical Test of Endophytic Bacteria

Endophytic bacteria obtained from the isolation were characterized by observing the shape, Gram characters and through the biochemical test including starch hydrolysis test, gelatin hydrolysis test, simon citrate agar, hydrogen sulfide, motility, catalase test and triple sugar test.

2.3. The Antagonistic Test of Endophytic Bacterial Isolates against Pathogenic Bacteria

Antibacterial activity of endophytic bacterial isolates was tested against pathogenic bacteria (Enteropathogenic Escherichia coli (EPEC), Staphylococcus aureus ATCC 29213, Listeria monocytogenes BTCC B693, Methicillin-resistant Staphylococcus aureus (MRSA) ATCC 43300, Staphylococcus epidermidis ATCC 12228, and Proteus vulgaris ATCC 13315). The tested bacteria was cultured in 5 mL of Nutrient Broth media and incubated at 37°C for overnight in shaking condition. The bacterial cultures were diluted 9 times of the initial volume by with sterile NaCl of 0.85%. As much as 3 mL of this solution was added to 17 mL of MHA Mueller Hinton Agar medium and then homogenized. The mixture was poured into sterile petri dish and waited until it became solidified. Paper discs containing 30 μL of endophytic bacteria were placed on surface media. Plates were incubated at 37 ± 2°C for 24 hours. The diameter of clear zone was measured to obtain microbial activity values.
2.4. Screening of Endophytic Bacteria against Phosphate Solvent Media
The endophytic bacteria were grown in a solid Pikovskaya medium [14] with a little of modification of media composition (g/L) of glucose (10.0), (NH$_4$)$_2$ SO$_4$ (0.5), Ca$_3$(PO$_4$)$_2$ (5.0), NaCl (0.3), MgSO$_4$.7H$_2$O (0.3), KCl (0.2), MnSO$_4$.4H$_2$O (0.03), FeSO$_4$.7H$_2$O (0.03), Yeast extract (0.5), agar (15.0), and distilled water (1000 mL). The bacteria were grown by using zig-zag streak method in middle part of the petri dish. The zig-zag method aims to observe the presence of a spectrum wide of endophytic bacteria colonies in dissolving phosphate. The incubation was done for 24 hours at a temperature of 25 ± 2˚C. Bacteria with a clear zone around the colonies were positive phosphate solubilizer.

3. Results

3.1. Isolation Characteristic of Endophytic Bacteria of T. barberi
Six culturable endophytic bacteria (EPS02, EPS21, EPS36, EPS37, EPS41 and EPS42) were successfully isolated from T. barberi root. Table 1 shows morphological and biochemical characteristic of each isolate. The isolates were then tested for their antibacterial and phosphate solubilization activity.

| Isolate Code | Cell Shape | Gram | Starch Hydrolysis | Gelatin Hydrolysis | Simon Citrate agar | Hydrogen Sulfide | Motility | Catalase | Triple Sugar Test |
|--------------|------------|------|-------------------|-------------------|-------------------|----------------|----------|----------|------------------|
| EPS02        | Bacilli    | Positive | +                  | +                 | -                 | -             | +        | +        | Yellow Pink      |
| EPS21        | Cocci      | Negative | -                  | +                 | +                 | -             | -        | +        | Yellow Pink      |
| EPS36        | Cocci      | Negative | -                  | +                 | +                 | -             | +        | +        | Yellow Pink      |
| EPS37        | Cocci      | Negative | -                  | -                 | +                 | -             | +        | +        | Yellow Pink      |
| EPS41        | Cocci      | Positive | +                  | +                 | -                 | -             | +        | +        | Yellow Yellow    |
| EPS42        | Cocci      | Positive | +                  | +                 | -                 | -             | +        | +        | Yellow Pink      |

3.2. Antibacterial activity of endophytes from T. barber
The test of antibacterial activity was conducted to determine the potential of endophytic bacteria of T. barber to inhibit the growth of tested bacteria. Each isolate showed different inhibition ability for tested bacteria capability; some endophytes inhibited two to three pathogens. The bacteria used were pathogenic for the humans. Antibacterial activity for each isolate is presented in Figure 1.
3.3. **Phosphate solubilizing activity of endophytes from T. barberi**

This test was conducted to observe the potential of isolates in dissolving phosphate, since it has been known that fern commonly grows in poor soil and sandy soil. The isolates were grown in Pikovskaya media containing Ca$_3$(PO$_4$)$_2$ for 24 hours. The ability of endophytic bacteria in solubilization phosphate was identified by formation of clear zone around the colony (Figure 2). Results showed that three of the six isolates did not form a clear zone which further indicated that the colonies were unable to solubilize phosphate (Table 2). EPS37 and EPS41 were observed to have a better ability to dissolve phosphate than EPS36. Importantly, formation of clear zone by the isolates could be observed in short incubation period; significant clear zone develop after 24 hours.

![Figure 2. Positive results of phosphate solubilization by bacterial isolates on Pikovskaya agar](image)

**Table 2. Phosphate solubilizing activity of endophytes from T. barber**

| No. | Bacterial isolate codes | Phosphatase |
|-----|-------------------------|-------------|
| 1   | EPS02                   | -           |
| 2   | EPS21                   | -           |
| 3   | EPS36                   | +           |
| 4   | EPS37                   | ++          |
| 5   | EPS41                   | ++          |
| 6   | EPS42                   | -           |

The activity was measured after 24 h of incubation at ± 28°C by measuring the clear zone: - , none activity; +,low; ++,high.

4. **Discussion**

We successfully isolated six bacterial isolates with antibacterial activities from *T. barberi* roots. These six endophytic bacteria isolates showed that they are capable of inhibiting the EPEC growth. Meanwhile, only EPS02, EPS36, and EPS37 which had antibacterial activity against *S. aureus* ATCC 29213.

Antibacterial activity against *S. epidermidis* ATCC 12228 was owned by EPS36, EPS41, while antibacterial activity against *P. vulgaris* ATCC 13315 was owned by EPS02 and EPS41. In addition, the test results that antibacterial activity against *L. monocytogenes* BTCC B693 was only owned by EPS32 isolate which also occur in ATCC 43300 MRSA inhibited by EPS21 isolate.

The measurement result of EPS36 isolates inhibitory zone against EPEC obtained the highest value of 8.6 mm. In addition, EPS36 and EPS37 endophytic isolates had antibacterial activity against *S. aureus* ATCC 29213 equals to 6.1 mm, followed by EPS02 isolates which had the best activity of 7.2 mm. EPS36 isolate also has antibacterial activity against *S. epidermidis* ATCC 12228 was 6.6 mm. Antibacterial activity from EPS02 against *P. vulgaris* ATCC 13315 was at 5.6 mm. In addition to
inhibit EPEC growth, EPS42 isolate also inhibit L. monocytogenes BTCC B693 was 5.7 mm. Interestingly, only EPS21 isolate had the ability to inhibit the pathogenic bacterium of MRSA ATCC 43300 which was 5 mm. According to Bibi et al. [15], inhibitory zones presented by endophytic bacteria with a value range of 7-9 mm are categorized as strong against pathogenic bacteria growth.

Human pathogenic bacteria still becomes a global issue of which the treatment solution is still continuously looked for. In this case, EPEC, S. aureus, L. monocytogenes, MRSA, S. epidermidis ATCC 12228, and P. vulgaris pathogens remains as the main focus. Exploration of the secondary compounds owned by endophytic bacteria can be used as an alternative solution. Based on the data obtained, endophytic bacteria from the T. barberi roots had inhibition ability against pathogenic bacteria. Endophytic bacteria show different activity in each pathogenic bacterium. Two endophytic bacterial isolates were categorized as strong against pathogenic bacteria, which are EPS36 strongly inhibited EPEC and EPS02 against S. aureus ATCC 29213.

Data on endophytic bacteria from ferns are still rarely found, especially in Tectaria barberries which have not been studied. The T. barberi collected from Sibolangit forest was the first one to be examined on its diversity of endophytic bacteria. Study on endophytic bacteria from Tectaria barberries was a part of an initial exploration in order to know its ability as an antibacterial agent. Das et al. [16] reported that the diversity of endophytic bacteria in uniformic Dryopteris ferns have antibacterial activity against L. monocytogenes, S. typhimurium, B. cereus, S. aureus, and E. coli O157: H7.

The existence of microorganisms in plant hosts can be affected by plant tissue compounds [17, 18]. Related to that endophytic bacteria can produce beneficial secondary metabolites which can be used by the industry of pharmaceutical since it certainly can produce the same metabolites as the host (plants) [17,18,19,20], endophytic bacteria from T. barberi will produce the same antibacterial bioactive compounds as T. barberries.

In addition to have antibacterial activity, endophytic bacteria from T. barberi also possess phosphate solubilization activity. Our research results showed that three out of six bacterial isolates were able to solubilize phosphate, namely EPS41, EPS36, and EPS37. This report has its own uniqueness, because in this case, the endophytic bacteria only needs 24 hours to solubilize phosphate, particularly for EPS41 and EPS37 isolates. Previous research result found that endophytic bacteria can dissolve phosphate within 5 days [21, 22, 23], 7 days [24] to 15 days of incubation [25]. A research result of Endophytic bacteria from T. barberi dissolving phosphate within 24 hours is the first time reported. It is a relatively fast time in dissolving phosphate affected by its gravel and rocky environments. Such habitat condition makes the bacterial around T. barberi roots adapt to use gravel and rocks as their source of calcium phosphate to grow. The pikovskaya media used also strongly supports endophytic bacteria T. barberi in dissolving phosphate since it contains Ca$_3$(PO$_4$)$_2$. Compared to the other endophytic bacteria taken from other plants, ferns are highly recommended to be used as a source of endophytic bacteria to grow a plant. The ability of EBF (Endophytic Bacteria Fern) in dissolving phosphate with a relatively faster time was very beneficial for plants to obtain good and efficient P (Phosphorus) source nutrients. In addition to nitrogen (N), phosphorus is also the most important key element to complement the plant nutrition. Its vital role is required by almost all metabolic processes such as photosynthesis, energy transfer, signal transduction, macromolecular biosynthesis and respiration [26].

Endophytic bacteria from T. barberi can be considered as the pioneer bacteria which symbiosis with the ferns tissue roots, therefore it can be an interesting research to be conducted. The existence of bacteria which can dissolve phosphate, with its habitat which is widely distributed in the soil [27], making it to be well symbiotic with the plant roots. In vitro, endophytic bacteria have the potential to increase the availability of organic and inorganic P (phosphorus) elements, through phosphatase synthesis by reducing soil pH, and chelating P from Al$^{3+}$, Fe$^{3+}$, Fe$^{2+}$ and Ca$^{2+}$ with the assistance of organic acids [27,28].

The prospect of endophytic bacteria from this study is very well to be applied in agriculture field. Phosphate dissolving activities in just 24 hours can quickly be absorbed by plants. As mentioned
before that P is the second most important nutrient for plants, after nitrogen. P in the soil is available as mineral salt or put into groups of organic compounds. Although the availability of P in the soil is abundant, mostly it is insoluble in the soil [29]. Therefore, endophytic bacteria can be used to help dissolve phosphates which can produce organic acids and acid phosphatase. It is in accordance with the statement from Illmer et al. [30] that the main mechanism for phosphate solubilization was the production of organic acids and acid phosphatase.

5. Conclusion
Six endophytic bacteria were successfully isolated from T. barber ferns roots. The isolates exhibited different extents of inhibition activity of tested pathogens. EPS36 has highest inhibition activity against EPEC (zone of inhibition 8.6 mm). The isolate also inhibited the growth of S. aureus and S. epidermidis by 6.1 and 6.6 mm, respectively. EPS36 and EPS37 isolates exhibited the same antibacterial activity against S. aureus ATCC 29213 (6.1 mm). EPS02 had the best activity against S. aureus (7.2 mm). EPS02 also showed antibacterial activity against P. vulgaris ATCC 13315. Among the six isolates, there isolates (EPS36, EPS37, and EPS41) exhibited phosphate solubilization activity after only 24 hours of incubation. These results indicates that potential of endophytic bacteria from fern T. barber both from the aspect of production of secondary metabolites and for biofertilizer need to be elucidated.

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