Antibacterial Activity of Bacterial Endophytes from Kupa Plant (Syzygium Polypephalum Miq. (Merr & Perry) Against Pathogenic Bacteria

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Abstract. The World Health Organization confirms that infectious diseases are normally treated using synthetic antibiotics. The bacterial endophytes from the tropical plant Syzygium polypephalum, locally known as Kupa, can be used as an alternative solution to reduce the utilization of synthetic antibiotics. The purpose of this study was to obtain one of the most potent bacterial endophytes to be used as antibacterial agents. In the present study, descriptive analysis was conducted consisting of bacterial isolation by pour plate method, bacterial identification with Vitek 2 compact BIOMERIEUX and antibacterial test by Kirby-bauer method. A total of 9 isolates of bacterial endophytes have been successfully obtained. From these isolates, a total of 4 species of endophytic bacteria were identified: Bacillus sp. (1), Bacillus sp. (2), Bacillus pumilus and Bacillus amyloliquefaciens. Our antibacterial tests revealed that Bacillus sp. (2) derived from the leaves appeared to be the most potent antibacterial isolates against pathogenic bacteria with 22 and 9 mm of inhibitory zone to Methicillin-resistant Staphylococcus aureus (MRSA) and to Bacillus cereus, respectively. On the other hand, endophytic isolate Bacillus sp (1) derived from stem was able to inhibit Klebsiella pneumoniae and B. cereus with inhibitory zones as much as 10 and 7 mm, respectively. This result strongly indicated that the antibacterial effect of bacterial endophytes from this study was species-specific and indeed the bacterial endophytes in this study could serve as a potential source of novel natural antibiotics.

1. Introduction
To date, the World Health Organization estimates that the infectious diseases are the world’s leading cause of death. Indeed, infectious diseases have caused death of approximately 13 million people worldwide each year, especially in developing countries as Indonesia [1].

Recently, Syzygium polypephalum Miq. (Merr & Perry), or locally recognized as Kupa, is an indigenous Indonesian plant. This plant has been considered as one of the medicinal plants due to its biological and chemical properties beneficial to cure several diseases. One of the bioactive compounds that was identified in this plant is flavonoid. This bioactive compound can be applied as antibacterial, wound infection inhibitor, anti-fungal, anti-viral, anti-cancer and anti-proliferative agents. In addition, flavonoid could also be utilized as anti-allergic, cytotoxic and anti-hypertensive [2] Apart from plant extracts, the bioactive compounds can be obtained from the bacterial endophytes.

Over the last decades, the bacterial endophytes are known as one of bioactive compounds producers, such as the secondary metabolite compounds with various biological activities [3]. These
microorganisms can live in either the vascular or in the intercellular space, roots, stems, leaves and fruit [4]. The bacterial endophytes have several advantages, such as their shorter life cycle than their host plants, which can save production time. Thus, the antibacterial compounds produced can be obtained on a large scale with less space. Another advantage of using bacterial endophytes is to preserve the sustainability of medicinal plants, especially rare or endangered species, hence not to be over-exploited.

Among the pathogenic bacteria, in the present study we mainly focus on four different bacterial species due to their importance pathogenicity and resistance to the synthetic antibiotics. *Pseudomonas aeruginosa* is bacteria that cause urinary tract infections, meningitis, diarrhea, entrokolitis necrosis and pneumonia [5] whereas *Klebsiella pneumonia* causes pneumonia, urinary tract infections and sepsis in patients with vulnerable immune system [6]. *Bacillus cereus* produces diarrhea-causing enterotoxins. On the other hand, *Methicillin-resistant Staphylococcus aureus* (MRSA) has a mutated gene that is resistant to almost all beta-lactam antibiotics. Meanwhile, *S. aureus* can cause septicemia, pneumonia, endocarditis, osteomyelitis, gastroenteritis and abscesses [7]. The present study aims to obtain the bacterial endophytes isolates from *S. polycephalum*, where the most potent isolate could be used as antibacterial agent against bacterial pathogens such as *P. aeruginosa, K. pneumoniae, MRSA* and *B. cereus*. This study could be as an alternative to over-used of synthetic antibiotics thus it can be medically accountable.

2. Materials and Methods

2.1. Isolation of bacterial endophytes

The isolation of bacterial endophytes was conducted by adapting the protocol from Krishnan et al., (2012). Briefly, the stems, fruits and leaves of Kupa plants were washed with clean running water. Afterwards, samples were cut into pieces with the size approximately 2-3 cm. These samples were then washed with sterile distilled water and soaked on 95% ethanol for 30 s, and then rinsed with sterile distilled water and dried. After, the samples were smoothed and isolated (1 g) for serial dilution until 10^-7. Furthermore, the last three dilutions were introduced into the petri dish containing the nutrient agar medium (NA) and incubated at 37 °C for 24 h [8].

2.2. Identification of bacterial endophytes

2.2.1. Macroscopic observation

On macroscopic observation, bacterial endophytes colonies were identified according to their shape, colour, elevation, edge and size.

2.2.2. Microscopic observation

The microscopic observation was performed by gram staining method. The positive gram bacteria were indicated by their purple colour. In contrast, the negative gram bacteria were indicated by their red colour.

2.2.3. Biochemical observation

The biochemical observation was conducted using Vitek 2-compact BIOMERIEUX. Briefly, bacterial endophytes isolates were inoculated on Trypticase Soy Agar (TSA), Blood Agar (BA) and Mac Concey Agar (MCA) medium. Furthermore, the positive gram bacteria were analysed using Vitex BCL cards. On the other hand, the negative gram bacteria were analysed with Vitex GN cards.

2.3. Identification of antibacterial activity

A total of one loop wire of bacterial endophytes isolates with 24 h lifetime were inoculated into 3 ml of NaCl physiology sterile to meet 0.5 Mc Farland turbidity with 2 times of dilution (1.5×10^6 CFU ml^-1). Afterwards, a total of 0.1 ml of suspension was inserted into a sterile vial containing paper discs, then incubated at 37°C for 24 h. Test on antibacterial activity was done by preparing 15 ml TSA medium in a sterile petri dish. Furthermore, a total of 0.1 ml of suspension of the tested bacterial strain with 24 h of lifetime with 0.5 Mc Farland turbidity (1.5×10^8 CFU ml^-1) were spread on the agar medium. Afterwards, the disc paper was placed on the agar surface and incubated at 37°C for 24 h prior to the measurement of bacterial inhibition zone. The measured parameter was the inhibition zone
(mm) formed around the disc paper. The results of the inhibition zone measurement were then categorized according to Pan et al. (2009) [8] as follows:

1. Inhibition zone diameter > 20 mm: very strongly sensitive,
2. Inhibition zone diameter 10-20 mm: strongly sensitive,
3. Inhibition zone diameter 5-10 mm: semi-sensitive,
4. Inhibition zone diameter < 5mm: resistant.

The percentage of inhibition (I%) was calculated according to the modified formula from Amadioha (2004) [9] as follows:

\[
I\% = \frac{dc - dt}{dc} \times 100 \quad \%
\]

Where dc represents the diameter of inhibition zone formed on control. Whereas dt represents the diameter of inhibition zone produced by the bacterial endophytes isolates from Kupa plant.

3. Results and Discussion

3.1. Isolation of bacterial endophytes

In the present study, the macroscopic observation of the bacterial endophytes isolates according to their colour, size, shape, edge and elevation is presented in Table 4.1.

| Kupa body parts | Isolates | Colour          | Size   | Shape     | Edge      | Elevation |
|-----------------|----------|-----------------|--------|-----------|-----------|-----------|
| Stems           | B 5-2    | Translucent white | Moderate | Amorphe | Diverged | Flat      |
|                 | B 6-3    | Milky white     | Small  | Spheric  | Flat      | Convex    |
|                 | B 7-4    | Translucent white | Large  | Amorphe | Smoothly jagged | Flat      |
|                 | D 5-1    | Milky white     | Moderate | Amorphe | Diverged | Flat      |
|                 | D 5-2    | Translucent white | Moderate | Amorphe | Smoothly jagged | Convex    |
|                 | D 6-3    | White           | Moderate | Spheric | Flat      | Convex    |
|                 | Bh-5     | Milky white     | Moderate | Spheric | Smoothly jagged | Convex    |
| Fruits          | Bh-6     | Milky white     | Moderate | Amorphe | Wavy      | Convex    |
|                 | Bh-8     | Yellowish white | Moderate | Amorphe | Wavy      | Flat      |

The microscopic observation of endophytic bacterial isolates by Gram staining technique at 1000 times magnification is presented in Table 4.2.

| Kupa body parts | Isolates | Cell shape | Gram staining |
|-----------------|----------|------------|---------------|
| Stems           | B 5-2    | Short bacillus | Positive     |
|                 | B 6-3    | Short bacillus | Positive     |
|                 | B 7-4    | Short bacillus | Positive     |
|                 | D 5-1    | Bacillus    | Positive     |
| Leaves          | D 5-2    | Long bacillus | Positive     |
|                 | D 6-3    | Bacillus    | Positive     |
3.2. Identification of bacterial endophytes

A total of 3 of bacterial endophytes isolates from Kupa stems were successfully identified. The isolate B 5-2 was allegedly identified as *Bacillus sp.* (1). On the other hand, bacterial endophyte isolate B 6-3 was fathomed to be *B. amyloliquefaciens* with 88% probability. Other isolates from the Kupa stems, the B 7-4 expected to be *B. amyloliquefaciens* with 91% of probability.

Endophytic bacterial isolates derived from Kupa leaves consist of 3 different isolates. The first isolate, D 5-1, was suspected to be *Bacillus sp.* (2). The second isolate, D 5-2, was predicted to be *B. amyloliquefaciens* with 91% probability. On the other hand, the isolate D 6-3 was predicted to be *B. amyloliquefaciens* with 91% of probability.

A total of 3 bacterial endophyte isolates from the fruits were identified. The isolate Bh-5 was predicted as *B. pumilus* with 90% probability, whereas isolate Bh-6 was suspected as *B. amyloliquefaciens* with 91% probability. Additionally, isolate Bh-8 was suspected as *B. amyloliquefaciens* with 85% probability.

3.3. Antibacterial activity of bacterial endophyte isolates

The results of antibacterial activity test can be seen in Figure 4.1 as follows

![Figure 4.1 Antibacterial activities of different bacterial endophyte isolates](image)

In this study, the antibacterial activities of bacterial endophyte isolates seemed to be species-dependent (Figure 4.1). The isolate D 5-1 had the highest activity against MRSA. However, this isolate appeared to be less sensitive against other pathogens. This is probably due to the different bioactive compounds as results from the bacterial endophytes secondary metabolites. In addition, the pathogenicity level of the bacterial strain tested could also influence the sensitivity of bioactive compounds from each isolates.

3.4. Antibacterial activity of bacterial endophyte isolates against *P. aeruginosa*

According to inhibition zone formed from the bacterial endophyte isolates against *P. aeruginosa*, it appeared that all isolates did not show any inhibition zone. Thus, it can be categorized as resistant. The isolate *Bacillus sp. (1)*, *Bacillus sp. (2)*, *B. amyloliquefaciens* and *B. pumilus* did not show inhibition zone at all around the disc paper. In addition to its resistance to endophyte isolates, *P. aeruginosa* was also resistant to antibiotic such as amoxicillin that was used as positive control.
Figure 4.2 Antibacterial activities of different bacterial endophyte isolates ((a) *Bacillus* sp. (1); (b) *Bacillus* sp. (2); (c) *B. pumilus*; (d) *B. amyloliquefaciens*; (e) Amoxicillin (positive control) and (f) control negative) against *P. aeruginosa*

To our knowledge, the resistance of *P. aeruginosa* to antimicrobial agents could be achieved through several mechanisms: (1) by reducing the permeability from the outer membrane, (2) by having a system that can automatically pump antibiotic out of the cell and (3) by producing the enzyme that can inactivate antibiotics [10].

3.5. Antibacterial activity of bacterial endophyte isolates against *K. pneumoniae*

In our study, results of the inhibition zone formed from the bacterial endophyte isolates against *K. pneumoniae* can be categorized as strongly sensitive. The inhibitory zone produced from bacterial isolates *B. pumilus* to *K. pneumoniae* was 12 mm or 20% higher than the inhibition zone from 30 µg of amoxicillin. Additionally, isolate *Bacillus sp.* (1) was able to produce 10 mm of inhibition zone or 0% to that of amoxicillin. Moreover, the inhibition zone formed on amoxicillin against *K. pneumoniae* was 10 mm.
3.6. Antibacterial activity of bacterial endophyte isolates against Methicillin-resistant S. aureus

According to classification by [8], our findings showed that the sensitivity levels of bacterial endophyte isolates to MRSA could be categorized as strongly sensitive. This result suggests that there was a difference in antibacterial activity. Indeed, the largest inhibition zone diameter was obtained in *Bacillus sp.* (2) with 22 mm or 144.44% to that of amoxicillin 30 µg. Furthermore, *B. amyloliquefaciens* isolate appeared to be the second with 12 mm or 33.33% compared to inhibition zone by amoxicillin. The inhibition zone formed by amoxicillin was 9 mm. We observed that the inhibition zone generated by *Bacillus sp.* (2) and *B. amyloliquefaciens* was greater than that of amoxicillin as positive control. Therefore, we may assume that the antibiotic produced from endophytic bacteria is more effective than the synthetic antibiotics.

These results are in accordance with the study conducted by Happy (2007), which demonstrates no significant difference in ampicillin, erythromycin, amoxicillin and generic tetracycline against *S. aureus*. Additionally, other study by Putri (2014) revealed that either amoxicillin or erythromycin did not produce inhibition zone that formed on the periphery of disc paper against *S. aureus*. This is probably related to the genes possessed by MRSA that can create resistance to beta-lactam antibiotics, such as amoxicillin.
3.7. *Antibacterial activity of bacterial Endophyte Isolates Against B. cereus*

From the inhibition zone formed against *B. cereus*, the antibacterial activities from the isolated bacterial endophytes could be categorized as sensitive. The inhibition zone produced from *Bacillus sp* (2) against *B. cereus* was 9 mm or 10% to that of amoxicillin 30 µg. Moreover, the isolate *Bacillus sp* (1) produced inhibition zone as much as 7 mm or 30% to that of amoxicillin. Indeed, the formed inhibition zone by amoxicillin against *B. cereus* was 10 mm.

![Figure 4.5 Antibacterial activities of different bacterial endophyte isolates](image)

(a) Bacillus sp. (1); (b) Bacillus sp. (2); (c) *B. pumilus*; (d) *B. amyloliquefaciens*; (e) Amoxicillin (positive control) and (f) control negative against *B. cereus*

4. *Conclusion*

In the present study, a total of 9 bacterial endophyte isolates were obtained from the Kupa plant *S. polycephalum*. These 9 isolates consist of 3 bacterial isolates from the stems, 3 isolates from the leaves and 3 other isolates from the fruits. Additionally, we demonstrated that bacterial endophyte isolates displayed inhibitory activity against the bacterial pathogens. The isolate *Bacillus sp.* (1) from the leaves produced inhibition zone as much as 10 and 7 mm against *K. pneumoniae* and *B. cereus*, respectively. Moreover, bacterial isolate *Bacillus sp.* (2) from Kupa’ stems produced 22 mm of inhibition zone against MRSA but only 9 mm to that of *B. cereus*. The isolate from Kupa fruits, *B. pumilus* generated 12 mm of inhibition zone against *K. pneumoniae*. On the other hand, other Kupa’ endophytic bacterial isolate *B. amyloliquefaciens* produced 12 mm of inhibition zone against MRSA. Our study suggests that the bacterial endophytes from Kupa plants may be used as potential candidate of antibacterial agents against the pathogenic bacteria.

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