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Isolation, Molecular Identification and Antibacterial Activity of Endophytic Bacteria from the Bark of *Plumeria acuminata*

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Abstract: Endophytic bacteria are able to produce bioactive compounds that are important for applications in the medical field. This study aims to isolate, identify and test the antibacterial activity of endophytic bacteria from the bark of *Plumeria acuminata* growing in the Mataram area. Isolation was carried out using four types of media, namely NA, TSA, MAC, and BHI. The well diffusion technique was used to test the inhibitory ability of the endophytic bacterial extract against the test pathogenic bacteria. The four test bacteria used were *S. aureus, B. cereus, P. aeruginosa, K. pneumoniae.* Isolates that showed inhibitory activity were then identified morphologically, biochemically and molecularly based on the 16S rRNA gene. The results of the isolation process obtained 35 colonies. where 9 of them inhibit the growth of test bacteria. There were 2 isolates that able to inhibit the growth of 4 types of test bacteria with an average clear zone diameter of very strong category (21–30 mm), namely isolates T1 and T2. The N2 isolate had the highest average inhibition zone compared to all isolates of endophytic bacteria, which was 32 mm, but was specific for *P. aeruginosa*. Two isolates, namely B1 and M3 gave various growth inhibitory effects with strong to very strong categories. Other isolates showed various antibacterial effects against at least 2 types of test bacteria. *S. aureus* was the most resistant pathogenic bacteria to the treatment of endophytic bacterial isolate extract. The cell shape of all endophytic isolates was streptobacillus with or without spores, and 50% of them were Gram-positive. Molecular identification based on the 16S rDNA gene grouped the 9 isolates into 2 main clusters, namely the genus *Bacillus* and the genus *Pseudomonas*. Isolates that were closely related to the genus *Bacillus* showed higher inhibitory activity than those of *Pseudomonas*. The results showed that isolates of endophytic bacteria from the bark of *P acuminata* have the potential as an important source of antibacterial substances.

Keywords: Endophytic bacteria; *P. acuminata;* Inhibition zone; 16S rDNA.

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Introduction

Today, the problem of antibiotic resistance has become a fundamental concern in the field of medicine. Pathogenic bacteria have been able to respond to the excessive use of antibiotics by producing their progenies that are no longer sensitive to certain antibiotics. Even against various groups of pathogens, including fungi and bacteria have been able to be resistant to several antibiotics (multidrug resistance) (Levy, 2002). Recently there has been an increase in the problem of multiresistant bacteria. As with methicillin resistant *S. aureus* (MRSA), this shows the increasing importance of research to find new sources of antibiotics that are able to overcome bacterial and fungal infections where the source of these antibiotics can come from a variety of biological resources.

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The new trend in the discovery of new drugs emphasizes the exploration of various antimicrobial compounds, which can be a source for treating various diseases (Nazar et al., 2009). Most of the chemical components derived from plants used as drugs or medicinal ingredients are secondary metabolites. According to Strobel & Daisy (2003), secondary metabolites produced from plant tissues have high biological activity. The use of herbal products as an alternative therapy for several diseases is increasingly widespread. This is because natural medicines have low side effects and are safe for long-term treatment (Ebadi, 2007).

One of the plants that is widely used as a source of traditional medicine is the white frangipani plant (*Plumeria acuminate*). P. acuminate is a plant originating from Central America, including the Apocynaceae tribe. *Plumeria* with the regional name (Indonesia) frangipani flower, is widely available in Mataram. *P. acuminata* species belong to the class Dicotiledonae, order Aposinales and family Apocynacea (Tjitrosupomo, 2000). Apart from being an ornamental plant, *P. acuminata* is also a medicinal plant. Some of the benefits of frangipani plants used as traditional medicinal ingredients are reducing pain due to swelling, antibacterial, toothache medicine, ulcers, warts, rheumatism, dysentery, fever, cough, and cracked soles (Mursito and Prihmantoro, 2011; Ashraf et al., 2012). Almost all parts of this plant can be used as traditional medicine, including bark, sap, roots, flowers and leaves. *P. acuminata* contains compounds of agoniadin, plumierid, plumeric acid, lipeol, serotinic acid, fulvoplumierin, saponins, flavonoids and polyphenols (Gupta et al., 2008; Gupta, & Yadav 2016).

Secondary metabolites contained in *P. acuminata* can inhibit the growth of *S. aureus* at a concentration of 25% with an inhibition zone of 12 mm (Wahyudi and Sukarjati, 2013). Antibacterial activity of ethyl acetate extract from the bark of *P. acuminata* against several pathogenic bacteria showed strong inhibitory activity. The ethyl acetate extract of *P. alba* bark contains alkaloids, terpenoids, and saponins (Sriwarthini, 2014; Yahya & Indies, 2014).

Recently, several endophytic bacteria and their bioactive compounds have been successfully used in the production of new commercial compounds and are very useful in the production of new drugs in medicine and crop protection in agriculture (Singh and Gaur, 2017; Mishra et al., 2018). Endophytic microbes are microorganisms that live in healthy plant tissues and become symbionts found in many plant species (Hallmann et al., 1997). This close relationship in plant tissue is a beneficial interaction for plants (Long et al., 2008). Endophytic microorganisms can be found in various plant tissues including seeds, ovules, fruits, stems, roots, tubers, roots and leaves but do not cause disease in these plants. Endophytic microorganisms have been isolated from various plants. Because endophytes can produce secondary metabolites with various properties that are good for their application, it has attracted the attention of many researchers to explore its potential and its use in biotechnology (Casella, et al., 2013; Kusari et al., 2013). Gandhi, et al, (2015) explained that endophytes can also produce various chemical compounds including secondary metabolites which are the same as those produced by the host plant.

The stem bark of *P acuminata* is often used as medicine. The bark of this plant may contain endophytic bacteria that can produce secondary metabolites that have antimicrobial activity similar to the properties of extracts from the host plant. The search for endophytic bacteria from the bark of the *P. acuminata* is expected to reveal the presence of endophytic bacteria capable of inhibiting the growth of pathogenic bacteria, as it has been proven that the secondary metabolite extract of the host plant has an inhibitory effect on various clinical isolates of pathogenic bacteria. This study aims to isolate, and identify endophytic bacteria from the stem bark of *P. acuminata,* and evaluate its in vitro antimicrobial properties.

Methods

Materials

P. acuminate bark, NA (Nutrien Agar) media, NB (Nutrien Broth) media, MHI (Muller Hinton Agar) media, MacConkey Agar media, TSA (Tryptose Soy Agar) media, BHI (Brain Heart Infusion) media, alcohol 70%, NaOCl 4%, tissue, Gram paint, aquadest, detergent. four type of test bacteria. Bacterial DNA isolation kit. Primer 63f : 5'- CAG GCC TAA CAC ATG CAA GTC – 3', primer 1387 r : 5'-GGG CGG WGT GTA CAA GGC– 3', master mix solution. agarose, EtBr, electrophoretic buffer.

Equipments

Electrophoresis equipment, PCR machine (thermocycler), 2 cc speute, 0.2 µl mess filter, test tube. petry disk, beaker glass, cotton swab, incubator. sterilizer, sterile catheter, shaker, laminar air flow, centrifuge, vortex, microscope, object glass. blue type. yelow type, micro pipette.

Microorganisms and media.

Four common antibiotic resistance human pathogens such as *S. aureus, P. aeruginosa, B. cereus, K. pneumoniae* were used to evaluate the antimicrobial activity of endophytic bacteria. The test bacteria were

obtained from the laboratory of the Biomedical Research Unit of the West Nusa Tenggara Provincial Hospital.

Endophytic bacteria isolation

The bark of *P. acuminata* is taken from the frangipani tree that grows in the city of Mataram. The stem bark pieces were cleaned of adhering dirt with running water followed by washing them in water with a few drops of detergent. Then the samples were immersed for 1 minute in 70% alcohol and washed twice with distilled water. Then it was immersed again in 4% sodium hypochlorite (NaOCI) for 5 minutes and rinsed with distilled water. Then the sample was dried in laminar air flow.

Four types of sterile media were prepared, namely Nutrient agar, Trypticase Soy agar, MacConkey's agar, and Brain Heart Infusion media. Using a sterile scalpel, the bark was cut by 1 x 0.5 cm and the pieces were planted on the media and incubated at 32ºC for 48 hours. The growing endophytic bacterial isolates were transferred to a nutrient agar slant tube and subcultured regularly every week and stored at 4ºC before use (Sahu et al., 2014)

Culture conditioning

Endophytic bacteria were grown in sterile 10 ml nutrient broth and incubated for 48 hours at 32ºC while shaking with a shaker at 150 cycles/minute. After incubation, the culture medium was centrifuged at 5000 g for 30 minutes, then the supernatant was separated and filtered. The filtered supernatant was used for antimicrobial activity and stored before use at 4ºC (Sunkar & Nachiyar, 2013).

Antibacterial activity test

Pathogenic bacteria with a concentration of 10^6 cells/ml were added to 2 ml of sterile 0.9% NaCl. 100µl of bacteria were spread on the Muller Hinton Agar plate evenly. Next, a well with a diameter of 8 mm on the plate that has been planted with the test bacteria was made. The well was filled 50 µl with the supernatant extract of endophytic bacteria isolate, and as a positive control the antibiotic ciprofloxacin was used. Antibacterial activity test by each isolate of endophytic bacteria against all test bacteria was carried out in 3 replications. The cultures were then incubated for 24 hours at 32ºC. The inhibition zone formed in the culture was measured and became data on the antibacterial activity of the endophytic bacteria isolates against the test bacteria.

Morphological and Biochemical Characterization of Isolates

Identification of endophytic bacteria was carried out on morphological, biochemical and molecular properties based on the 16S rDNA gene. Morphological identification was carried out by Gram staining, biochemical identification was carried out by TSI, Urea, Simon citrate, Motility, glucose, sucrose, lactose, maltose, MR, VP, catalase, coagulase tests (Vasanthakumari, 2009).

Molecular Identification of Bacterial Isolates DNA Extraction

One ose of sample was added with 200 of DNA Zole and then vortexed for 1 minute. 100 µl of 100% ethanol was added and allowed to stand for 5 minutes. Centrifuge 4000 g for 4 minutes. Washed 2 times with 200 µl of 80% ethanol and allowed to stand for 4 minutes. After washing it was centrifuged at 4000 g for 2 minutes. Then dissolved in 40 µl of distilled water and stored at – 20ºC until use

PCR Process

The primer used is Primer 63f : 5'- CAG GCC TAA CAC ATG CAA GTC – 3', Primer 1387 r : 5'- GGG CGG WGT GTA CAA GGC – 3'. The PCR mix reaction consisted 2x PCR Master mix solution (10 µl), DNA template (1-2 µl), Primer f (1 µl), Primer r (1 µl), aquadest (6-7 µl), so the total volume was 20 µl. Then the PCR tube was inserted into the PCR machine (BioRad). DNA amplification was carried out using the MyCycler (Bio Rad) tool. Pre-PCR was carried out for 10 minutes at a temperature of 94°C and followed by 38 PCR cycles with denaturation steps for 30 seconds at a temperature of 94°C, annealing for 30 seconds at a temperature of 55°C, and extension for 45 seconds at a temperature of 72°C. After 35 cycles were exceeded, Post PCR was performed for 10 minutes at a temperature of 72°C and 1 minute at a temperature of 20°C. The PCR product was electrophoresed to see the success rate of the process. At this stage, 4 µl of PCR product was added with 2 µl of loading buffer (Bromophenol-blue and cyline cyanol), and electrophoresed on 2% agarose gel in TAE buffer (0.5 gram agarose plus 50 ml TAE) which had been filled with 4 µl ethidium bromide (EtBr). Electrophoresis was carried out at a voltage of 100 V and a current of 400 A for 30 minutes. The marker used was 1000 bp DNA Ladder (Invitrogen). The results of the electrophoresis were visualized under ultraviolet light and photographed using a Gel Doc (Bio Rad). PCR products showing DNA bands were sequenced. The sequence data were edited using Clustal W in the MEGA 5 program and the results were then compared with the sequences at GenBank using the BLAST search facility found on the NCBI website (http://www.ncbi.nlm.nih.gov). To determine the phylogenetic tree, the results of each sequence were edited and arranged into a contiguous sequence using Sequence Scanner and Bioedit software. Furthermore, for making alignment and phylogenetic tree using MEGA5 Software. The phylogenetic tree to determine the genetic relationship between isolates was compiled using the neighbor joining method.

Data Analysis

The data collection was carried out by observing the morphological and biochemical characteristics of the isolates. The diameter of the clear zone formed (in MHA media) in the inhibitory activity test by endophytic isolates was measured in mm, and the interpretation of the average level of inhibition (antibacterial activity) of the isolates against the test bacteria referred to the category according to Morales et al. (2003). The 16S rDNA gene sequence data from endophytic bacterial isolates were analyzed using MEGA 5 software. The BLAST method was used to access the 16S rDNA gene database on *Genbank*, which is available on the NCBI website (http://www.nebi.nml.nih.gov). The phylogenetic tree of the isolates was constructed using the neighbor joining method.

Result and Discussion

Endophytic bacteria isolation

The results of the initial isolation process obtained 35 colonies of endophytic bacteria that grew from the bark of *P. acuminata* explant grown on 4 types of media. Details of the number of colonies obtained by media type are presented in **Table 1**.

Tabel 1. The number of endophytic bacterial colonies obtained from explants of *P. acuminata* stem bark using 4 types of isolation media

No.	Type of Media	Number of colonies	Percentage
1	Brain Heart infusion	5	14.3 %
2	Nutrien Agar	8	22.9 %
3	MacConkey's Agar	10	28.6 %
4	Trypticase Soy Agar	12	34.3 %
Total Numberof colonies		35	100 %

The highest number of colonies was obtained from TSA media (12 colonies) followed by MAC media (10 coloniest), NA (8 colonies), and then BHI media with 5 colonies. The use of these 4 types of media is intended to obtain many colonies of endophytic bacteria. These results indicate that the isolation media used can affect the type and number of colonies that grow from plant explants on the isolation media. Ngamau *et al.* (2009) used 5 different media to get as many endophytic bacterial colonies as possible. The results of the initial screening of 35 colonies showed that only 9 colonies were able to inhibit the growth of pathogenic bacteria.

Antibacterial test of the endophytic isolates

Of the 35 colonies obtained, there were 9 colonies that were able to inhibit the growth of pathogenic bacteria, *S. aureus*, *B. cereus*, *P. aeruginosa, and K. pneumoniae*. The test was carried out with 3 replications to get the average value of the clear zone which was a representation of the ability of endophytic bacteria to inhibit the growth of the test bacteria. Some examples of in vitro test results for the inhibitory activity of the isolates are presented in **Figure 1.**

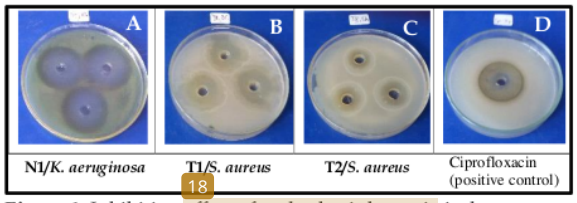

Figure 1. Inhibition effect of endophytic bacteria isolate extract on the growth of some pathogenic bacteria in the form clear zone on MHA media. Isolate N1 inhibition activity on the growth of *P. aeruginosa* (A), isolate T1 inhibition on the growth of *S. aureus* (B), isolate T2 inhibition on the growth of *S. aureus* (C), Cyprofloxasin antibiotic inhibition on the growth of *K. pneumoniae* as positive control (D)

The complete results of the antibacterial activity test of 9 isolates of endophytic bacteria isolated from the bark of *P. acuminata* against 4 types of test bacteria are presented in **Figure 2**.

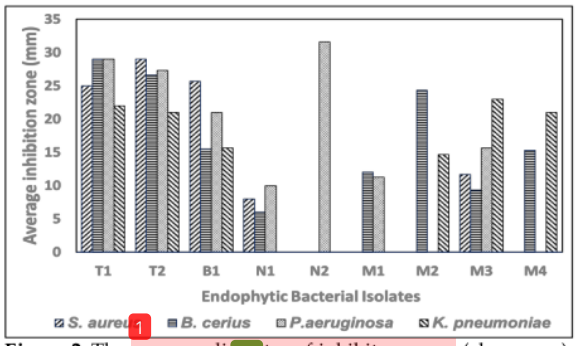

Figure 2. The average diameter of inhibitory zone (clear zone) of endophytic bacteria isolated from the stem bark of *P. acuminata* against the human pathogenic bacteria. (*S. aureus, B. cereus, P. aeruginosa* and *K. pneumoniae).* Inhibition activity category (Morales, et al., 2003) : weak activity (<5 mm), moderate (5–10 mm), strong (11-20 mm), and very strong. (21–30 mm).

The Evaluation of the level of inhibitory activity of endophytic isolates against test bacteria was carried out according to the categories of Morales et al, (2003), namely weak activity (<5 mm), moderate (5–10 mm), strong (11-20 mm), and very strong. (21–30 mm). There were 4 isolates capable of inhibiting the growth of 4 types of test bacteria with an average diameter of the clear zone with a very strong category (21–30mm), namely isolates T1 and T2. Isolate B1 gave various

growth inhibition effects on the tested bacteria, with very strong inhibition categories against *S. cerius* (25 mm), *P. aeruginosa* (21 mm) and moderate effect on both of *B. cerius* and *K. pneumoniae* with an average The average diameter of the inhibition zone is about 15 mm. N1 isolate had moderate antibacterial effect (5-10 mm) against *S. aureus, B. cereus, P. aeruginosa* and did not inhibit the growth of *K. pneumoniae*. The N2 isolate had the highest average inhibition zone compared to all endophytic bacterial isolates, which was 32 mm, but this was specific for *P. aeruginosa*, while it had no effect on the other 3 test bacteria. The M1 isolate had a strong effect (approximately 12 mm) against both *B. cereus and P. aeruginosa*. M2 isolate showed a very strong inhibitory activity against B. cerius (24.33 mm), and a strong effect against *K. pneumoniae* (14.67 mm). The M3 isolate had very strong against *K. pneumoniae* (23 mm). M4 isolate gave a very strong antimicrobial effect against *K. pneumoniae* and a strong category against *B. cerius* with inhibition zone diameters of 21 mm and 15.33 mm, respectively.

The average diameter of the clear zone shown by each isolate varied greatly, this was due to the different abilities of each endophytic isolate and also the different responses given by the test bacteria (**Figure 1**). Some endophytic isolates did not have an inhibitory effect on the growth of the test bacteria. The formation of a clear zone is due to the metabolism of endophytic isolates that produce secondary metabolites. It is possible that some of the secondary metabolites produced by this endophytic bacterial isolate are the same as those produced by the host plant, *P. acuminata*. Genetic transfer can occur from the host plant to its endophytic microbes causing secondary metabolites produced by the host plant to also be produced by the endophytic symbionts (Ludwig-Müller, J., 2015; Tiwari & Bae., 2020).

Some of the isolates were classified as broad-spectrum antibacterial activity, because they were able to inhibit the growth of all test bacteria, both Gram negative and Gram positive with a very strong category, namely > 20 mm, such as isolates T1, T2, B1 and M3. Endophytic bacteria isolate N2, which is closely related to *Bacillus cereus* strain PM-01 hg 1 (**Table 3**), had the highest ability to inhibit the growth of the tested pathogenic bacteria (**Figure 2**). This may be because *Bacillus* has spores and is stable which in turn produces secondary metabolites to protect its life, besides that *B. cereus* is also insensitive to penicillin. It has been found that *Bacillus* produces antifungal agents such as hydrolytic enzymes and fungicins (Yao et al., 2003; Lin et al., 1999). The isolate M1 which is closely related to the *Alcaligenes faecalis* strain (ZD02) is capable of inhibiting the growth of two target pathogenic bacteria, namely *B. cereus and P. aeruginosa* with a strong category. Isolate B1 which closely related to *Pseudomonas putida* strain (II-B) was also able to inhibit the growth of all test bacteria. The isolate N1 belonging to the genus *Pseudomonas* was able to inhibit 3 target test bacteria (*S. aureus, B. cereus, P. aeruginosa*). Meanwhile, the isolate M4 belonging to the genus *Pseudomonas* (**Table 3**) was only able to inhibit the growth of *B. cereus and K. pneumoniae*. Genus *Pseudomonas* is capable of producing alpha amylase in addition to cellulose and proteases (Liu et al., 2011). The findings in this study showed that almost all endophytic bacterial isolates had strong (11-20 mm) and very strong (21-30 mm) inhibition categories as shown in **Figure 2**, and in addition almost 50% of the isolates had broad-spectrum antibacterial properties because it can inhibit the growth of both Gram-positive and Gram-negative bacteria.

The ability of *P. acuminata* endophytic bacteria to inhibit the growth of pathogenic bacteria is thought to be because it can produce the same secondary metabolites as their host. The secondary metabolites of *P. acuminata* include iridioids, tannins, alkaloids, plumierid, fulvoplumierin plumieric acid, lupeol-acetate, oxymethyldioxy cinnemic acid, lupeol, acetil lupeol, alpha-amyrin, beta amyrin, cerotic-acid Plumericine, isoplumericine, beta-dighydroplumeric-acid, Quercetin, Quercetinglycoside, phenyl-ethyl-alcohol, kaempferol, 1-(+)-bornesitol, linalool, citronellol, farnesol, gerniol (Ramesha and Srinivas, 2014).

P. acuminata methanol extract has antimicrobial activity against Gram-positive bacteria, as well as Gram negative bacteria (Gupta et al., 2008). Likewise, the sap of *P. acuminata* flowers also has the ability to inhibit the growth of *Shigella dysentery* (Ardila, 2013). Meanwhile, ethanol extract of the bark of P. acuminata was also able to suppress the growth of *B. cereus and S. aureus* (Sriwarthini, 2014). These findings show that there is concordance that the antimicrobial properties of endophytic bacteria isolated from the stem bark of *P. acuminata* are also related to the ability of the host plant extracts to act as antimicrobials in inhibiting the growth of various pathogenic microbes.

Biochemical and morphological characterization

Biochemical tests were carried out with TSI media, urea, simon citrate, motilation, glucose, sucrose, lactose, fructose, maltose, mannitol, MR, VP, catalase and coagulase. The results of biochemical and morphological characterization are in **Table 2**.

Tabel 2. Biochemical tests Gram stain results and the shape of endophytic bacteria isolated from the bark of *P. acuminata*

No	Test	Endophytic bacteria isolates								
		T1	T2	B1	N1	N2	M1	M2	M3	M4
1	TSI	B/A	B/A	B/A	B/A	B/A	B/A	B/A	B/A	B/A
2	Urea	–	–	–	–	–	–	–	–	–
3	Simon Citrate	–	–	–	–	–	–	–	+	–
4	Mot	+	+	+	+	+	+	+	+	+
5	Glu	+	+	+	+	+	+	+	+	+
6	Sucr	+	+	+	+	+	+	+	+	+
7	Lac	+	+	–	–	–	–	–	+	–
8	Malt	–	–	+	+	+	+	+	–	+
9	MR	+	+	+	+	+	+	+	–	+
10	VP	–	–	+	+	–	–	–	–	+
11	Cat	+	+	+	+	+	+	+	+	+
12	Coagulase	+	+	+	+	–	+	+	–	+
13	Gram type	+	+	–	–	+	–	+	+	–
14	Cell shape	streptobacilli, spores terminal	streptobacilli, spores terminal	streptobacilli	Streptobacilli	streptobacilli, spores central	Streptobacilli	streptobacilli, spores central	streptobacilli, spores terminal	streptobacilli

Note : A : Acid; B : Base; Cat : Catalase; TSI : Triple Sugar Iron; Mot : Motility; Glu : Glucose Sucr : Sucrose; Lak : Lactose; Malt : Maltose; MR : Methyl Red; VP : Voges Proskauer.

Molecular identification

The process of molecular identification begins with the isolation of genomic DNA from endophytic isolates, followed by a PCR process using primers 63f and 1387r, 2% agarose gel electrophoresis, PCR product sequencing and bioinformatics analysis based on the 16S rDNA database of bacterial genomes at *Genbank*. A PCR product with a size of about 1300 bp is seen on the gel (**Figure 3**)

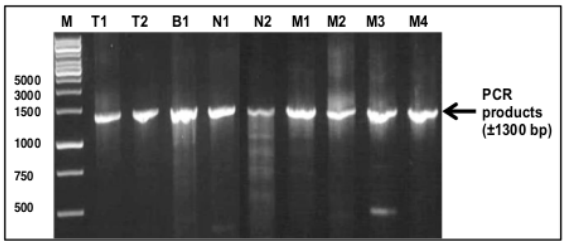

Figure 3. Results of amplification of 16S rRNA using 63f and 1387r primers run on electrophoresis at 2% agarose gel from 9 endophytic bacteria isolated from *P. acuminata*; M : Molecular marker (1 kb DNA Ladder); Arrow indicates the PCR product size (1300 bp).

Table 3. The results of identification of 9 endophytic bacteria isolated from the bark of *P. acuminata* based on the sequence of the 16S rRNA genes using BLAST-N

Bacterial isolates (Strain ID)	16s rDNA closest relative	Maximal score	Query cover	Identity
T1	*Bacillus cereus strain* NC7401	948	96%	99%
T2	*Bacillus cereus* strain WPD 16S	852	81%	81%
B1	*Pseudomonas putida* strain II-B	2150	98%	97%
N1	*Pseudomonas putida* Strain JT-K21	2161	94%	97%
N2	*Bacillus cereus* strain PM-01 hg 1	1003	98%	99%
M1	*Alcaligenes faecalis* ZD02	2255	98%	99%
M2	*Bacillus cereus* strain HYM76	1295	64%	97%
M3	*Bacillus cereus* strain 03BB108	1814	76%	98%
M4	*Pseudomonas plecoglossicida* strain BOAKS 441	2257	99%	98%

Based on the results of the BLAST-N analysis on the NCBI site by querying the results of DNA sequence of isolates, the species with the closest percentage of similarity identity to the 16S rDNA sequence of the isolates was obtained (**Tabel 3**). In this case, isolate T1 has 99% similarity with *B. cereus* strain NC7401, while isolate M4 has 98% similarity with *P. plecoglossicida* strain BOAKS 441. Meanwhile, the alignment and phylogenetic tree were made using MEGA5 software. The phylogenetic tree was constructed using the

neighbor joining method and the results are as shown in **Figure 4**.

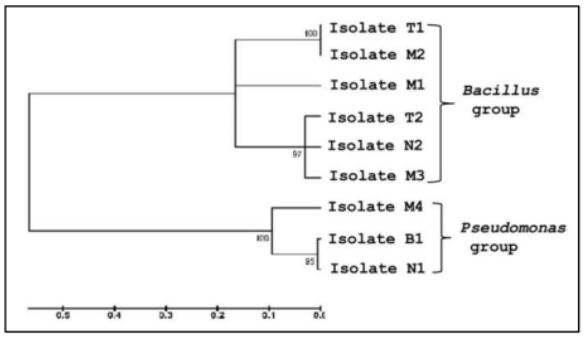

Figure 4. Phylogenetic tree of endophytic bacteria isolated from the bark of *P. acuminata* and their relationshp among them. The dendrogram was constructed using the Neighbor-Joining method.

Based on the results of molecular analysis based on the 16S rDNA gene above, 9 endophytic bacteria that were able to inhibit pathogenic bacteria isolated from the bark of *P. acuminata* were divided into 2 clusters, namely the genus *Bacillus* (isolates T1, T2, N2, M2 and isolate M3) and the genus *Pseudomonas* (isolates B1, N1 and M4), and there is one member of the genus *Alcaligenes*, namely isolate M1. In this study, based on the percentage of the number of isolates that were able to inhibit the test bacteria and the level of antimicrobial activity, there is an indication that closely related isolates to the genus *Bacillus* showed a better antibacterial activity the test bacteria than those of the genus *Pseudomonas*.

Conclusion

In this study, 35 isolates were obtained, of which only 9 isolates had the ability to inhibit the growth of the test bacteria with varying degrees of inhibition activity from moderate to very strong level. This varied inhibition was thought to be caused by the difference in the number and types of antibacterial compounds produced by each isolate. Two isolates were able to inhibit the growth of 4 types of test bacteria with a very strong category, namely isolates T1 and T2. N2 isolate had the highest average inhibition zone compared to all isolates of endophytic bacteria. *S. aureus* was the most resistant bacteria to the endophytic bacterial extract isolated from the stem bark of *P. acuminata*. The cell shape of all endophytic isolates was streptobacillus with or without spores, and 50% of them were Gram-positive. Molecular identification based on the 16S rDNA gene, 9 isolates of endophytic bacteria were grouped into 2 main clusters, namely genus *Bacillus* and genus *Pseudomonas*.

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