Antibacterial activity of *Achromobacter sp.* and *Bacillus sp.*, bacterial endophytes derived from Mangrove *Ceriops tagal* (Perr.) C.B.Robb

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Abstract. A total of 95 bacterial endophytes have been isolated from mangroves plant in Bengkalis coastal area, Riau Province, Indonesia. Mangrove plant samples were identified as *Bruguiera sp.* and *Ceriops tagal* (Perr.) C.B.Robb. Antagonism study showed that 4 out of 95 isolates were possessed antibacterial activity based on their ability to inhibit the growth of *Vibrio alginolyticus* and *Staphylococcus aureus*; while none of them was able to inhibit *Escherichia coli*. According to the results of 16s rRNA genes amplification, the closest species relative of the 4 active isolates was *Achromobacter insolitus* (isolate no. 34), *Bacillus siamensis* (isolate no. 39), and *Bacillus subtilis* (isolate no. 88 and 89). This is the first report on bacterial endophytes associated with Mangrove in Riau Province, Indonesia.

1. Introduction
Antibiotic resistance, the ability of specific bacteria to survive in the presence of an antibiotic resulting in drug inefficiency and persistent infections, has been studied for a long time. More than a hundred different types of antibiotics discovered have been used to treat numerous infectious diseases; however, drug resistance does exist. The world faces the crisis in antibiotic discovery as it is predicted by 2050, the increased number of resistant pathogens will cause 10 million deaths per year. Not only for humans, but the antibiotic resistance is also a big issue in the environment since the increase of its number has been accelerated in recent years caused by the discharge of antibiotics into the environment [1-3]. It is given that new antibiotics are needed to combat drug-resistant pathogens. Numerous studies on plants and their derivatives against hundreds of microbial strains have reported that they found remarkable antimicrobial potential [4-10].

Plants and their associated microorganisms have evolved together to adapt to a given environment. One species of the plant could be a host of thousands of microorganisms which are categorized as epiphytes and endophytes. Recently, endophytes - microorganisms that live in the inner tissues of plants without causing any external symptoms have been under increased investigation due to their
complex relationship with the host. The presence of endophytes in the host plants provides several benefits, particularly growth promotion and protection from pathogens. It could be due to the metabolites of endophytes that have been reported to inhibit a number of microorganisms. Several studies reported the ability of endophytes to produce host-derived metabolites of importance such as alkaloids, terpenoids, phenolic, steroids, and tannins that serve as a potential candidate for antimicrobial, anticancer, and other biological activities [11-14].

Mangroves are an important ecosystem. They provide crucial ecosystem services, including fisheries, shoreline shields, and bioremediation of wastes [15]. Since they strive under extreme conditions and straddle the terrestrial and aquatic biomes, mangrove environments are a potential source to explore new microbiota with extensive applications in pharmaceutical science [16; 17]. Indonesia has 27% of the world's total mangrove forest, equivalent to 4.25 million ha [18], and 143 billion ha are in Riau Province. Considering the virtual lack of studies on endophytes derived from Mangrove in Riau Province, it reported here for the first time the bacterial endophytes from mangrove plant at Bengkalis coastal area.

2. Methodology

2.1. Sampling site and bacterial endophytes isolation
Fresh and healthy roots, stem barks, and leaves of mangrove plants were taken randomly from Sei Pakning (Siak Regency at 1°20’55”N, 102°9’33”E) and Tenggayun Beach (Bengkalis Municipality at 1°32’3”N, 101°54’58”E), and then were sent to Riau University Herbarium for identification. Some roots were brought to the laboratory for endophytic bacteria isolation in separate sterile polyethylene bags. Each root sample was washed in running tap water. The surface of each sample was sterilized by immersing each of them in 70% ethanol (2 minutes), soaked in 5.25% peroxide acid (2 minutes), rinsed in sterile distilled water (three times), and surface-dried with sterile filter paper. Aliquots of the last sterile distilled water used in the final rinse were inoculated onto the surface of Nutrient Agar (NA) plates supplemented with ketoconazole 0.3g/100ml for 18-24 h to confirm that the surface sterilization is successful. Each of those surface-sterilized samples was cut into fragments and each fragment was put onto the surface of ketoconazole-supplemented NA plates and then incubated at 37 °C for 24-48 h. Any bacterial growth around the incubated fragment samples was then taken and purified (streaked) onto other sterile NA plates, incubated overnight at 37 °C, all single colonies with different characters were taken and maintained as bacterial endophyte isolates.

2.2. Antagonism test
All endophytes isolated were screened for the antagonism in vitro against Escherichia coli, Staphylococcus aureus, and Vibrio alginolyticus. One colony of each bacterial endophyte was transferred (drawn as a circle of 6mm diameter) on the surface of Mueller Hinton Agar containing each pathogenic bacterium (OD 0.1) and incubated for 24-48 h. All treatments were performed in triplicate. Inhibition zones produced around each endophyte were measured and bacterial endophytes showing good antibacterial activity were then subjected to macroscopic, microscopic analysis, gram staining, and molecular species identification.

2.3. 16S rRNA Gene Amplification and Sequencing
DNA template of each active isolates was extracted using Presto™ Mini gDNA Bacteria kit. The amplification of 16S rRNA genes was carried out with two primers (24F/1541R) and performed in 50 μL reaction volumes of 5.0 μL 10x PCR Buffer, 2 μL of DNA, 2.5 μL of 2 mM dNTPs, 0.2 μL of 5 U/μL Taq Polymerase, 1 μL of each primers and 38.3 μL of sterile distilled water. The reaction was carried out in an amplification condition of 95 °C at 5 min for pre-denaturation followed by 35 cycles of denaturation (95 °C; 1 min), annealing (50 °C; 1 min) and extension (72 °C; 1 min and 30s). The final extension was at 72 °C (10 min). Gel electrophoresis was carried for the PCR products using 1% agarose with TBE buffer at 80 V for 25 min. PCR product was then sent for sequencing at Genetica Science Indonesia. Finally, the 16s rRNA gene sequence homology was then analyzed using the Mega BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence homology was compared with
that of other microorganism sequences of GenBank. The phylogenetic tree was constructed by the neighbor-joining method using the MEGA software version 6.06 based on 1000 bootstraps.

3. Results and Discussion

A total of 95 bacterial endophytes have been isolated from root samples of Mangroves which were identified as *Ceriops tagal* (Perr.) C.B.Rob and *Bruguiera* sp. All isolates were screened for their ability to inhibit the growth of three pathogens studied. Out of 95 isolates, we found 30 isolates (Table 1) gave inhibition zones. There were 24, 4, and 5 isolates that were able to inhibit the growth of *Vibrio alginolyticus*, *Escherichia coli* dan *Staphylococcus aureus*, respectively. All positive isolates were then used for antagonism test and the result showed that isolate number 34, 88, and 89 were active to inhibit *V. alginolyticus*; isolate number 34 and 39 were produced inhibition zones against *S. aureus*; while none of them was active against *E. coli* (Table 1; Figure 1).

### Table 1. Bacterial endophytes, sources, and their antibacterial activities

| No. | Isolate No. | Mangrove samples | Antibacterial screening | Antagonism result |
|-----|-------------|------------------|-------------------------|-------------------|
|     |             |                  | *V. alginolyticus* | *E. coli* | *S. aureus* | (diameter of inhibition zone; mm) |
| 1   | 1           | Bruguiera sp.    | +                      | -         | -         | n.d        | n.d        | n.d        |
| 2   | 2           | Bruguiera sp.    | +                      | -         | -         | n.d        | n.d        | n.d        |
| 3   | 4           | Bruguiera sp.    | +                      | -         | -         | n.d        | n.d        | n.d        |
| 4   | 5           | Bruguiera sp.    | +                      | -         | -         | n.d        | n.d        | n.d        |
| 5   | 6           | Bruguiera sp.    | +                      | -         | -         | n.d        | n.d        | n.d        |
| 6   | 7           | Bruguiera sp.    | +                      | -         | -         | n.d        | n.d        | n.d        |
| 7   | 11          | Bruguiera sp.    | +                      | -         | -         | n.d        | n.d        | n.d        |
| 8   | 23          | Bruguiera sp.    | +                      | -         | -         | n.d        | n.d        | n.d        |
| 9   | 26          | Bruguiera sp.    | +                      | -         | -         | n.d        | n.d        | n.d        |
| 10  | 27          | Bruguiera sp.    | -                      | +         | -         | n.d        | n.d        | n.d        |
| 11  | 31          | Bruguiera sp.    | +                      | +         | -         | n.d        | n.d        | n.d        |
| 12  | 33          | Ceriops tagal    | +                      | -         | -         | n.d        | n.d        | n.d        |
| 13  | 34          | Ceriops tagal    | +                      | -         | +         | 11.3±0.5   | n.d        | 9.7±0.4    |
| 14  | 37          | Ceriops tagal    | +                      | -         | -         | n.d        | n.d        | n.d        |
| 15  | 39          | Ceriops tagal    | -                      | -         | +         | n.d        | n.d        | 10±0.5     |
| 16  | 41          | Ceriops tagal    | -                      | -         | +         | n.d        | n.d        | n.d        |
| 17  | 52          | Ceriops tagal    | +                      | -         | -         | n.d        | n.d        | n.d        |
| 18  | 53          | Ceriops tagal    | +                      | -         | -         | n.d        | n.d        | n.d        |
| 19  | 62          | Bruguiera sp.    | +                      | -         | -         | n.d        | n.d        | n.d        |
| 20  | 65          | Bruguiera sp.    | +                      | -         | -         | n.d        | n.d        | n.d        |
| 21  | 68          | Bruguiera sp.    | +                      | -         | -         | n.d        | n.d        | n.d        |
| 22  | 69          | Bruguiera sp.    | +                      | -         | -         | n.d        | n.d        | n.d        |
| 23  | 71          | Bruguiera sp.    | -                      | +         | -         | n.d        | n.d        | n.d        |
| 24  | 72          | Ceriops tagal    | -                      | -         | +         | n.d        | n.d        | n.d        |
| 25  | 73          | Ceriops tagal    | +                      | -         | -         | n.d        | n.d        | n.d        |
| 26  | 74          | Ceriops tagal    | +                      | -         | -         | n.d        | n.d        | n.d        |
| 27  | 81          | Ceriops tagal    | +                      | -         | -         | n.d        | n.d        | n.d        |
| 28  | 88          | Ceriops tagal    | +                      | -         | -         | 9.1±0.5    | n.d        | n.d        |
| 29  | 89          | Ceriops tagal    | +                      | -         | -         | 8.9±0.1    | n.d        | n.d        |
| 30  | 92          | Ceriops tagal    | -                      | -         | +         | n.d        | n.d        | n.d        |

n.d: not-detected
Figure 1. Overnight inoculated sterilized-surface sample (A), Single colonies (B), inhibition zones (C)

According to the antagonism data, this study selected four isolates i.e., isolate no.34, 38, 88, and 89 as active isolates to be identified further for morphology and molecular species identification. Isolate number 34 is only the one with coccus morphology, while others were bacilli. Interestingly, all of them were isolated from Ceriops tagal (Perr.) C.B.Rob; and isolate number 34 showed a potential source of broad-spectrum antibiotics since it could inhibit the growth of both gram-negative and gram-positive bacteria.

The electrophoresis product of amplified DNA was found ~1500 bp. Species identification was carried out by comparing DNA sequences obtained to the DNA sequences in the NCBI GenBank Database (Table 2). According to the Max score and identity, the closest species of isolate no.34, 39, 88, and 89 are Achromobacter insolitus, Bacillus siamensis, Bacillus subtilis, and Bacillus subtilis, respectively. The nearest phylogenetic neighbour sequences for 16s rRNA gene sequences of the 4 isolated bacterial endophytes were shown in Figure 2.

Table 2. Closest species relative of the four active isolates based on sequence similarity using BLAST program in the GenBank database

| Description of species | Max score | Query cover | E-value | Per. Ident |
|------------------------|-----------|-------------|---------|------------|
| Isolate No. 34         |           |             |         |            |
| Achromobacter xylosoxidans strain ES-6 | 2382 | 100% | 0.0 | 97.14% |
| Achromobacter insolitus strain Y2P1 | 2382 | 100% | 0.0 | 97.14% |
| Achromobacter denitrificans strain USDA-ARS-USMARC-56712 | 2378 | 100% | 0.0 | 97.07% |
| Isolate No. 39         |           |             |         |            |
| Bacillus amyloliquefaciens strain X030 | 2616 | 100% | 0.0 | 100.00% |
| Bacillus velezensis strain WRN014 | 2616 | 100% | 0.0 | 100.00% |
| Bacillus subtilis isolate K1 | 2616 | 100% | 0.0 | 100.00% |
| Bacillus siamensis | 2616 | 100% | 0.0 | 100.00% |
| Isolate No. 88         |           |             |         |            |
| Bacillus velezensis strain SRCM100072 | 2616 | 100% | 0.0 | 100.00% |
| Bacterium CulalenE33 | 2616 | 100% | 0.0 | 100.00% |
| Bacillus amyloliquefaciens subsp. plantarum YAU B9601-Y2 | 2616 | 100% | 0.0 | 100.00% |
| Bacillus subtilis strain ZJ06 16S | 2616 | 100% | 0.0 | 100.00% |
| Bacillus siamensis | 2611 | 100% | 0.0 | 99.93% |
| Isolate No. 89         |           |             |         |            |
| Bacillus siamensis strain SCSIO 05746 | 2531 | 100% | 0.0 | 98.76% |
| Bacillus velezensis strain SRCM100072 | 2531 | 100% | 0.0 | 98.76% |
| Bacterium CulalenE33 | 2531 | 100% | 0.0 | 98.76% |
| Bacillus amyloliquefaciens subsp. plantarum YAU B9601-Y2 | 2531 | 100% | 0.0 | 98.76% |
| Bacillus subtilis strain ZJ06 | 2531 | 100% | 0.0 | 98.76% |
Isolate no. 34, which was able to inhibit both gram-positive and gram-negative bacteria studied, has the closest species relative with *Achromobacter insolitus*. The genus *Achromobacter*, of the family *Alcaligenaceae*, originally contained a single type species, *Achromobacter xylosoxidans*. Through times, several species were added to the family based on a polyphasic taxonomy study [19,20], including *Achromobacter xylosoxidans* and *Achromobacter denitrificans*. Further study is needed to extract and characterize any compounds produced by each isolated bacterial endophyte.

4. Conclusions

Bacterial endophytes of mangrove plants are alternative sources in finding some antibacterial compounds because 4 of 95 endophytes isolated could inhibit the growth one to two of pathogens studied. According to the results of 16s rRNA genes amplification, the closest species relative of the four active isolates was *Achromobacter insolitus* (isolate no. 34), *Bacillus siamensis* (isolate no. 39), and *Bacillus subtilis* (isolate no. 88 and 89).

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