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# Antibacterial Production by Endophytic Bacteria from *Catharanthus roseus* in East Timor Against Methicillin-Resistant *Staphylococcus aureus*

**Hildegardis Missa[1*], Sardina Ndukang[1], Aloysus Djalo[1], Getrudis W. Nau[1], Ari Susilowati[2], Anselmus Boy Baunsele[3], Americo Dos Santos[4]**

[1] Biology Education Study Program Faculty of Teacher Training and Education Widya Mandira Catholic University. Jl. San Juan No 1. East Penfui, Kupang District, East Nusa Tenggara 85361, Indonesia.
[2]Biology Study Program, Faculty of Math and Natural Sciences, Sebelas Maret University, Jl. Ir Sutami 36, Kentingan, Jebres, Surakarta, Central Jawa 57126, Indonesia.
[3]Chemistry Education Study Programs Faculty of Teacher Training and Education Widya Mandira Catholic University. Jl. San Juan No 1. East Penfui, Kupang District, East Nusa Tenggara 85361, Indonesia.
[4]Biology Study Program Faculty of Education, East Timor Cofee Institue, Jl. Ermera, Gleno, Timor Leste

*Coreesponding Author: hildegardismissa@unwira.ac.id

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**Abstract.** Methicillin-resistant Staphylococcus aureus (MRSA) refers to a group of bacteria that cause infections and present a challenge in treatment due to their resistance to several antibiotics. Therefore, research is needed to discover endophytic bacteria from *Catharatus roseus* capable of producing new antibiotics. This study aims to identify endophytic bacteria from *C. roseus* plants originating from Timor Island, as producers of effective antibacterial compounds against MRSA. Antibacterial activity tests were conducted on two types of test bacteria, namely MRSA and *Escherichia coli*. DNA extraction was performed using the PrestoTM Mini dDNA kit Bacteria, and amplification of the 16S rRNA gene was carried out. The research found bacterial isolates showing morphological similarities to *Bacillus sp*. Screening results indicated that four bacterial isolates exhibited high potential antibacterial activity against MRSA, as evidenced by the formation of inhibition zones with diameters of approximately 25 mm-35 mm. Amplification of the four endophytic bacterial isolates from *C. roseus* identified them as *Paenibacillae* FaCH2, *Bacillae* BoCH3, *Aneurinibacillae* BoCH5, *Aneurinibacillae* BiCH8, which are new species based on 16S rRNA gene similarity of less than 97%. In conclusion, endophytic bacteria from *C. roseus* producing antibacterial compounds against MRSA have been successfully identified. This is beneficial to society because the antibacterial compounds produced can serve as a basis for developing new drugs that are effective against MRSA infections, considering the increasing antibiotic resistance against MRSA.

**Keywords:** Antibacterial; *Catharatus roseus*; Endophytic Bacteria; MRSA; Timor

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## INTRODUCTION

The issue of bacterial resistance to antibiotics worldwide represents a significant global health concern (Sukertiasih et al., 2021). Antibiotics have long been used to prevent and treat disease (Gultom et al., 2023). The mechanism for developing bacteria to resist the effects of antibiotics has been used since antibiotics were introduced or are called resistant bacteria. The swift rise in bacterial resistance to antibiotics is becoming a critical global health issue (Zhou et al., 2022). Several factors can cause the problem of bacterial resistance to antibiotics namely the ease with which people can get antibiotics and the lack of government health supervision of the public regarding irrational use of antibiotics such as choosing antibiotics that do not suit the patient's condition and inappropriate antibiotic prescribing patterns (Rahman et al., 2023).

*Staphylococcus aureus* bacteria are normal flora in the human body that can be found in the nose, skin, throat and mouth. *S. aureus* have strain resistant to penicillin class antibiotics or commonly known as Methicillin-resistant *Staphylococcus aureus* (MRSA) (Rahman et al., 2023). Infection caused by MRSA has become a health problem that is of concern not only in

several countries but in all parts of the world and to date some of the problems of bacterial resistance have never been completely resolved (Mena et al., 2023). Explorations needed to discover new sources of natural antibiotics. Currently microorganisms are a rich source of many compounds useful in pharmaceutical and agricultural treatment. Hence, scientists are concentrating on acquiring novel microorganisms from diverse traditional medicinal plants which have been extensively used by indigenous communities for generations to treat ailments. This constitutes the main objective in the quest for discovering new bioactive compounds namely by identifying endophytic bacteria.

Endophytic bacteria are saprophytic bacteria that reside within and are linked to plant tissue without inducing any disease symptoms in the plants but they can form a mutualistic symbiotic relationship in obtaining nutrients from the results of plant metabolism and can protect plants against insects and pathogenic microbes (Ayilara et al., 2023). Some endophytic microorganisms in plants on the island of Timor can live in extreme conditions with high levels of stress, with unstable light conditions and air temperatures making it possible to discover unique metabolisms that can be utilized to produce new metabolites which are of course different from the endophytic microorganisms that have been discovered. previously. Endophytic microorganisms bacteria for example can aid the immune system by producing antibiotics and other active substances. Hence, endophytic microorganisms discovered in Timor Island, East Nusa Tenggara, present considerable potential for uncovering new compounds with intriguing biological activities such as antimicrobial, antifungal, antibiofilm, antioxidant, and anticancer properties (Mena et al., 2023).

*Catharatus roseus* is one of the plants used by the Timorese people as traditional medicine. The antibacterial properties of *C. roseus* are attributed to its flavonoid compounds which have been demonstrated to disrupt the permeability of bacterial cell walls, microsomes, and lysosomes through interactions with bacterial DNA (Pham et al., 2020). On the island of Timor *C. roseus* plants are planted in home gardens as ornamental plants. Even though the benefits of this plant are widely known by most people, research and information regarding endophytic bacteria and their potential are not yet known. Judging from the use of the *C. roseus* plant as a traditional medicine for the people of Timor which can cure various diseases such as toothache, fever, as an antibacterial, and antioxidant (Tang et al., 2022), Hence, it is crucial to investigate the potential bioactive compounds derived from endophytic bacteria inhabiting *C. roseus* plants in Timor, East Nusa Tenggara. This represents a vital criterion in the endeavor to discover novel bacterial reservoirs proficient in generating secondary metabolites as antibiotics and offering crucial alternatives in combating drug resistance levels in pathogens like MRSA.

The aim of this study is to identify endophytic bacteria from the *C. roseus* plant native to Timor Island, evaluate the antibacterial potential of the endophytic bacteria against MRSA, and determine the phylogenetic relationships between the endophytic bacteria from *C. roseus* on Timor Island and previously discovered endophytic bacteria. The benefits of this research include expanding scientific knowledge about the diversity and potential of endophytic bacteria from *C. roseus* as a source of new antibacterial compounds, providing scientific data on endophytic bacteria that are effective against MRSA, generating alternative solutions for treating MRSA infections which are a global health issue due to antibiotic resistance and utilizing the biodiversity of Timor Island particularly the *C. roseus* plant, as a potential source for health product development. Additionally, it can promote innovation in the fields of biotechnology and pharmaceuticals through the exploration of endophytic microorganisms for the production of antibacterial compounds.

## METHODS

### Sampling

*C. roseus* leaves as the source of endophytic bacteria were taken from three locations namely Fatubena, Bonen and Binilaka, Oeltua Village, Kupang Regency, East Nusa Tenggara Province. The sampling method employed was purposive sampling. The sampling was determined based on differences in location and the presence of samples at each sampling (Figure 1).

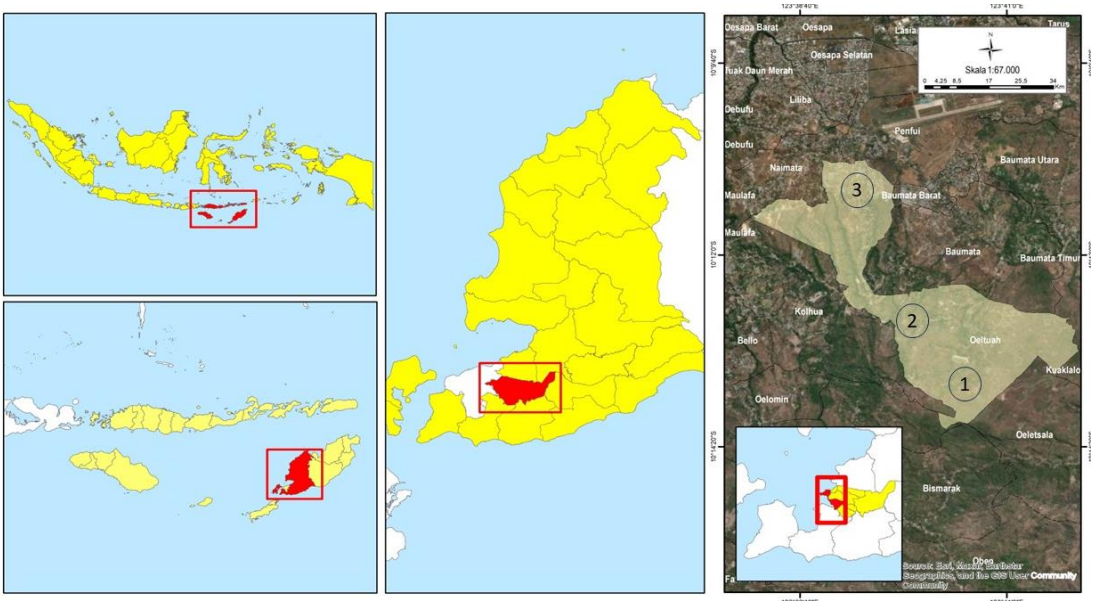


**Figure 1.** Study location on Timor Island, Oeltua Village, Kupang Regency, East Nusa Tenggara, Indonesia. 1). Fatubena 2) Bonen 3) Binilaka

The collected samples comprised 5-10 leaf segments from *C. roseus* which were placed into plastic containers and appropriately labeled with details regarding their sampling location. At each sampling site two different *C. roseus* plants were taken. Various samples were collected from nearby locations and subsequently transported to the laboratory for the identification of endophytic bacteria. The bacterial cultures used for testing against MRSA and *E. coli* were sourced from the medical laboratory at Nusa Cendana University, Kupang. These cultures were stored on nutrient agar slants (Himedia).

**Isolation of *Catharatus roseus* Endophytic Bacteria**

The samples were washed under running water for ± 5 minutes until clean, then continued by soaking the samples using 70% ethanol solution for 5 minutes (Amatullah et al., 2023), then soaking it again using sodium hypochloride solution (4%) for 3 minutes then washing the sample again using sterile distilled water 3 times for 2 minutes to remove the sterilizing agent and then the samples were dried. The sterile sample was weighed at 5 grams then scraped using a mortal and then put in 45 ml of sterile distilled water, diluted with a $10^{-3}$ dilution series and then inoculated on Miller-Schroth (MS) media and incubated at room temperature for 3-4 days. Following the incubation period, the proliferation of endophytic bacteria became evident across the entire surface of the MS medium. Bacterial colonies exhibiting diverse morphologies were isolated and preserved as pure cultures using nutrient agar medium.

**Screening of endophytic bacteria as antibacterial against Methicillin Resistance *Staphylococcus aureus* (MRSA) and *Escherichia coli.***

Pure cultures of endophytic bacteria are inoculated on Nutrient Broth (NB) media and shaker at a speed of 170 rpm for 3-4 days (Missa et al., 2023). The removal of cell biomass from NB medium containing the secondary metabolites of endophytic bacteria (supernatant) was accomplished through centrifugation at a speed of 5000 rpm for 1 hour. The supernatant is utilized for screening endophytic bacteria that produce antibacterial compounds (Hadian & Supronienė, 2023). 40 µl of supernatant was applied onto a sterile paper disc and air-dried on a sterile watch glass for 30 minutes to remove excess water. Subsequently, the test bacteria were cultured on Nutrient agar (NA) medium via pour-plating onto various petri plates. This was achieved by transferring 1 ml of the test bacterial suspension into 20 ml of molten NA maintained at 50°C, with gentle shaking to ensure thorough mixing of the bacteria and NA, Once the paper disc inoculated with the supernatant has been compressed, it is positioned on the surface of the medium. Inoculation is conducted at a temperature of 37°C for 24 hours or until a distinct zone forms around the paper discs. For screening endophytic bacteria, sterile NB served as the negative control, while Chloramphenicol was used as the positive control.

The inhibitory potential was assessed by measuring the diameter of the clear zone with a ruler (Rahardiyan & Moko, 2023). Antibacterials are categorized as weak if the resistance is 1.4 – 6.2 mm, medium category if the resistance is 6.3 – 10.3 mm, strong category if the resistance is 10.4 – 26.8 mm and very high category if the resistance is ≥ 26.8 mm (Mahdi et al., 2022).

**Genomic DNA Extraction**

To isolate bacterial DNA, 1.5 ml of bacteria was transferred into a tube, centrifuged at 10,000 rpm for 2 minutes, and then DNA extraction was performed using the PrestoTM Mini gDNA kit for bacteria (Geneid) (Amatullah et al., 2023).

**Amplification of the 16S rRNA Gene by PCR**

The isolated bacterial DNA using a Biorad PCR machine in 30 µl of solution consisting of 15 µ PCR Master Mix. Nexpro, 1.5 µl DNA Template sample (100 ng /µl), 11.5 Water, 3 µl Pimer (5 pmol each forward and reverse primer) (Mukaromah et al., 2023). The primer used is 16S 27F 5' AGAGTTTGATC{AC)TGGCTCAG3', and 16S 1492R 5' TACGG(CT)TACCTTGTTACGACTT3'. Amplification was carried out by setting a pre-denaturation temperature of 95℃ for 2 minutes, *annealing* at 50°C for 30 seconds, and *extension* at 72° for 2 minutes. Next, the post *elongation* process was carried out at 72° for 7 minutes. The PCR results were then electrophoresed on 1% agarose (Giri et al., 2020). The PCR outcomes were subsequently subjected to sequencing using the sequencing service provided by 1stBASE Laboratories Sdn Bhd.

**Data analysis**

The 16S rDNA underwent bioinformatic analysis using a nucleotide sequence alignment tool known as Basic Local Alignment Search Tool Nucleotides (BLAST-N), accessible on the National Center for Biotechnology Information (NCBI) website (Mukaromah et al., 2023), to ascertain the resemblance between each potential endophytic bacteria from *C. roseus* and previously identified endophytic bacteria cataloged in the GenBank 16S rRNA gene database. The connection between potential endophytic bacterial species from *C. roseus* and previously identified endophytic bacteria was examined by generating a phylogenetic tree utilizing the Molecular Evolutionary Genetics Analysis (MEGA) 20 software.

## RESULTS AND DISCUSSION

**Isolation of Endophytic Bacteria in *Catharatus roseus* with antibacterial activity against MRSA**

Isolation of endophytic bacteria in *Catharatus roseus* leaves from Timor, East Nusa Tenggara Province, Indonesia from three sampling locations, 23 isolates were found that grew on MS media and each isolate was obtained from six research samples, including those presented in table 1.

**Table 1 .** Isolation of endophytic bacteria C. roseus from Timor, East Nusa Tenggara

| Sampling Place | Number of Isolates / Station | | Isolate which has antibacterial activity |
|---|---|---|---|
| | I | II | |
| Fatubesi | 5 | 3 | 1 |
| Bonen | 5 | 5 | 2 |
| Binilaka | 2 | 3 | 1 |
| Amount | 12 | 11 | 4 |

The exact number of endophytic bacteria in plants cannot be determined, but these bacteria can be detected by isolating them on agar medium. Endophytic bacteria can grow with an incubation period of 2 days, this is supported by research done by Gultom et al., (2023) which states that the incubation time of a minimum of 2 days aims to ensure that the bacteria growing are endophytic bacteria, not contaminating bacteria. The results of macroscopic observations of pure isolates of *C. roseus* leaf endophytic bacteria showed that the morphological characteristics of the colonies showed the most similarities to *Bacillus colonies.sp*. According to (Magdalena, 2020) the morphological characteristics of colonies *Bacillus sp* namely round shape, yellowish white color, flat edges, rough texture and flat or convex elevation .

Endophytic bacterial isolate *C. roseus* was then tested for antibacterial activity against MRSA and *E. coli* . This test is carried out by growing endophytic bacteria and test bacteria in the same petri dish. The antibacterial activity of endophytes against the test bacteria is shown by the presence of a clear zone that appears to be away from the paper discs treated with endophyte bacteria. This test uses two controls, namely the positive control using chloramphenicol antibiotics 0.01 g/10ml and negative control using sterile NB media. Chloramphenicol was employed as a positive control due to its broad-spectrum antibiotic properties, capable of inhibiting both gram-

positive and gram-negative bacteria. The bacteria employed for testing include gram-positive bacteria, specifically MRSA, and gram-negative bacteria, particularly *E. coli*. The antibacterial test results revealed that four isolates of endophytic bacteria showed potential antibacterial activity, as evidenced by the formation of an inhibition zone around the paper disc containing bacterial cultures. Of the four potential endophytic bacteria, one isolate, namely (FaCH2), was isolated in samples taken in Fatubena, two isolates (BoCH3 and BoCH4) in Bonen and one isolate (BiCH8) in Binilaka, where The BiH8 bacterial isolate has the greatest inhibitory power, namely 35 mm for the MRSA test bacteria and the FaCH2 isolate has the highest inhibition zone, namely 25 mm for the *E. coli* test bacteria, thus the endophytic bacterial isolate on *C. roseus* leaves has the potential to be an antibacterial source against bacteria which are resistant and non- resistant some of which are presented in table 2.

Table 2 shows that each isolate code has a different inhibitory power. According to Tashan et al., (2021) the longer the bacterial fermentation time, the more secondary metabolites will be produced, which will affect the diameter of the inhibition zone. The presence of an inhibition zone suggests that these endophytic bacteria may possess antibiotic-producing potential and engage in secondary metabolism or produce extracellular compounds that act as toxins capable of inhibiting or killing bacterial growth. Endophytic bacterial isolates from *C. roseus* leaves, namely BiCH8, BoCH5, and BoCH3, exhibit significant potential for inhibiting or eradicating MRSA bacteria. BiCH8 displays the highest effectiveness with an inhibitory zone diameter of 35 mm, followed by BoCH5 with 30 mm, and BoCH3 with 25 mm. Meanwhile, bacteria with the FaCH2 isolate code have antibacterial potential in the strong category. Endophytic bacteria were detected with *E. coli* as the test bacteria in three isolates: FaCH2 showed an inhibition zone diameter of 25 mm, BoCH3 had an inhibition zone diameter of 19 mm, and BoCH5 exhibited an inhibition zone diameter of 21 mm. Meanwhile, isolate BiCH8, with an inhibition zone diameter of 13 mm, fell into the medium category. In contrast to the study conducted by (Tashan et al., 2021) on the antibacterial effects of endophytic bacteria on turmeric plants, the results revealed that the maximum inhibition zone diameter reached 13.76, classified as strong inhibition. Apart from that, research conducted by Safara et al., (2022) showed the highest inhibitory power obtained from oil palm leaf endophytic bacteria, namely 13 mm in the medium category. Therefore, the endophytic bacterial isolate from *C. roseus* in Timor exhibits a highly potent inhibition zone, with a diameter of 35 mm, indicating promising potential for the development of new antibiotics. The findings from the antibacterial activity assessment are displayed in Figure 2.

**Table 2.** Endophytic bacteria extracted from *Catharatus roseus* leaves exhibit antibacterial properties against MRSA and *Escherichia coli*.

| Isolate Code | Clear Zone Diameter (mm) | | | |
|---|---|---|---|---|
| | MRSA | Growth Barrier Response | *Escherichia coli* | Growth Barrier Response |
| FaCH2 | 25 | Strong | 25 | Strong |
| BoCH3 | 27 | Very strong | 19 | Strong |
| BoCH5 | 30 | Very strong | 21 | Strong |
| BiCH8 | 35 | Very strong | 13 | Currently |
| Chloramphenicol | 25 | Very strong | 21 | Strong |
| Sterile Broth Nutrients | 0 | None | 0 | None |

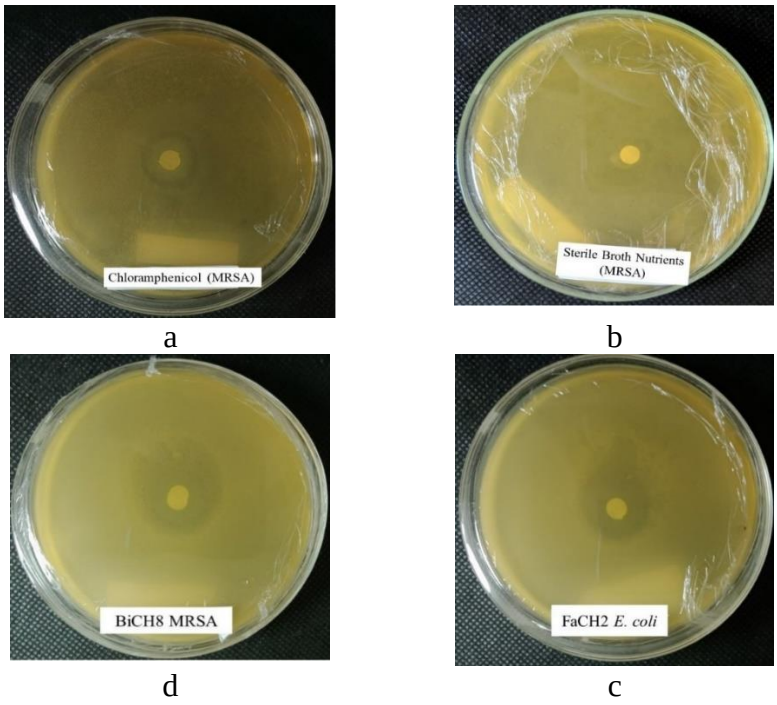


**Figure 2** . The clear zone shows antibacterial activity formed by the endophytic bacteria *C. roseus* (a. Chloramphen MRSA test bacteria, b. Sterile Broth Nutrients MRSA test bacteria, c. BiCH8 isolate of MRSA test bacteria and d. FaCH2 *E. coli* test bacteria )

**Amplification of the 16S rRNA coding gene in *Catharatus roseus* leaf endophytic bacteria**

Amplification of the 16S rRNA coding gene involves replicating the genomic DNA strand obtained from DNA isolation using a PCR cycle to acquire the 16S rRNA coding gene. The primers employed are universal primers designed for amplifying the 16S rRNA gene, targeting an amplicon of 1500 base pairs. In this study, primers 27F and 1492R were used with an amplicon target of around 1300 bp (Amatullah et al., 2023). The coding genes in bacteria exhibit significant diversity due to their presence in prokaryotic organisms. Primers are the most important component in a PCR reaction because they can determine the region of the genome that will be amplified (Rahardiyan & Moko, 2023). Figure 3 illustrates the agarose gel electrophoresis procedure conducted with a 0.5% (w/v) concentration, lasting 45 minutes at 85 volts and a current of 300 mA, aimed at determining the sizes of the amplified DNA fragments of the 16S rRNA gene obtained via PCR.

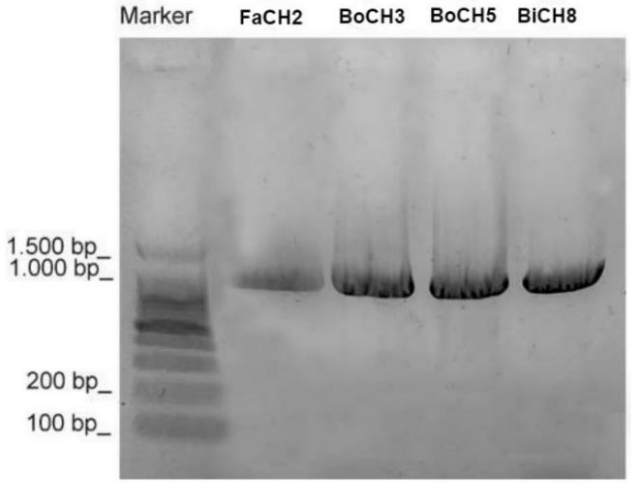


**Figure 3.** PCR product of the 16S rRNA gene encoding Isolation of *C. roseus* endophytic bacteria (FaCH2, BoCH3, BoCH5 and BiCH8 isolates )

**Table 3 .**Similarity of the endophytic bacteria *C. roseus* based on the 16S rRNA coding gene using the BLAST program

| Isolate | Most Similar Species | No. Access | % Similarity | Identity of Potential Endophytic Bacteria |
|---|---|---|---|---|
| FaCH2 | *Paenibacillusdendritiform* Strain T168 16S ribosomal RNA, partial sequencing | JX535565.1 | 91. 81 | *Paenibacillae* FaCH2 |
| BoCH3 | *Bacilluscereus* Strain JCM 2152 16S ribosomal RNA partial sequencing | MT256066.1 | 92. 65 | *Bacillae* BoCH3 |
| BoCH5 | *Aneurinibacillusmigulanus*NBRC strain 16S ribosomal RNA partial sequences | MF040218.1 | 8 4.76 | *Aneurinibacillae* BoCH5 |
| BiCH8 | *Aneurinibacillusmigulanus* Strain NBRC 15520 16S ribosomal RNA, partial sequences | OP558488.1 | 90.65 | *Aneurinibacillae* BiCH8 |

Figure 3 shows that the bacterial 16S rRNA gene is well amplified, indicated by the presence of bands that are bright and thick and parallel to each other. This indicates that the primer used attaches to the DNA template at the optimal temperature for primer annealing. The appearance of the band indicates that the primer used is specific, attaching only to the expected site (Wang et al., 2023). The size of PCR can also be evaluated by comparing the migration length of known-size markers with the migration length of DNA bands. Proper primer selection and *annealing* temperature are key factors influencing the quality of molecular detection using PCR method (Iman et al., 2023). The sample resulting from the amplification can proceed to the sequencing stage.

DNA sequences can be utilized to identify genes or other DNA fragments and determine their functions by comparing them with known DNA sequences. Identifying bacterial species using the 16S rRNA gene involves comparing the sequencing results with the reference sequences stored in the GenBank database (Sune et al., 2020). The results obtained from the examination of the 16S rRNA coding gene through BLAST are presented in table 3.

According to genetic alignment, the FaCH2 isolate had a sequence similarity of 91.81% with the *Paenibacillus dendritiform* bacteria strain T168 that has been assigned by NCBI GeneBank with accession number JX535565.1. *Paenibacillus dendritiform* bacteria are pattern-shaped bacteria, and they form colonies with complex and dynamic architecture.This is in line with research conducted by Mena et al., (2023) who discovered that the *Paenibacillus dendritiform* bacteria isolates forms a branched colony morphology, at the end of which it splits or is called the T morphotype showing thinner branches with a distinctive curved pattern so that this bacterium is said to have a unique colony morphology and different phenotypic characteristics. The bacteria Paenibacillus dendritiform comprises facultative anaerobic organisms capable of forming endospores. Initially categorized within the Bacillus genus, they were subsequently reclassified as a distinct genus in 1993 (Yadav et al., 2021). This bacterium has been found in diverse habitats such as soil, water, rhizosphere, plants, and insect larvae (Guan et al., 2023), It generates a range of extracellular compounds including enzymes that break down polysaccharides and proteases. These compounds have the ability to catalyze a wide array of synthetic reactions across different sectors, spanning from cosmetics to biofuel production. Additionally, it generates antimicrobial compounds that impact a broad range of microorganisms including fungi, soil bacteria, bacteria that cause plant diseases, and even anaerobic pathogens. (Mena et al. 2023). *Paenibacillus dendritiform*r bacteria are reported to have antibacterial ability against the *E. coli* (MTCC) bacteria, *S. aureus* (ATCC 25923), *Bacillussubtilis* (ATCC 6633), *Candida albicans* (MTCC 224) (Singh et al. 2019), Genus *Pseudomonas, Bacillus, Enterococcus,* and *Lactococcus* (Mena et al., 2023).

The BoCH3 isolate shares a sequencing similarity of 92.65% with the Bacillus cereus isolate JCM 2152 strain. This bacterium is gram-positive, rod-shaped, facultative anaerobic, motile, and exhibits beta hemolysis. It is commonly found in soil, water, air, and plants. This bacterium has also been widely reported to

have antibacterial abilities against *E. coli* and *Salmonella enteritidis* bacteria (Abreu et al., 2023. Meanwhile, BoCH5 and BiCH8 isolates have similarities to *Aneurinibacillus migulanus* bacteria. The potential of this bacterium is significant due to its ability to produce Gramicidin S antibiotic, which has efficacy against various gram-positive and gram-negative bacteria, as well as certain fungi and oomycetes, including some Ascomycota and Basidiomycota (Guan et al., 2023).

**Sequence the 16S rRNA coding gene *Catharatus roseus* endophytic bacteria**

Phylogenetic relationship based on the 16S rRNA gene were analyzed using a phylogenetic tree with mega 10 program. This analysis aims to construct precise relationships between organisms and estimate differences that occur from an ancestor to its descendants (Amatullah et al., 2023). The 16S rRNA gene is commonly utilized in the development of phylogenetic trees because the precise comparison of nucleotide base sequences among rRNA molecules facilitates the identification of diversity. In this study, the initial phase in constructing a phylogenetic tree involves aligning the sequences of FaCH2, BoCH3, BoCH5, and BiCH8 isolates with multiple isolates obtained from GenBank using Clustal W. Subsequently, the phylogenetic tree is constructed utilizing the neighbor-joining method, Bootstrap with 1000 iterations, and the p-distance method. The neighbor-joining method utilizes sequences which, when combined, offer the most accurate estimation of branch length, closely resembling the actual distance between the sequences. According to (Sadiqi et al., 2022), a nucleotide base similarity below 97% suggests distinct species. The findings from the phylogenetic tree analysis reveal the identification of bacterial species and genera through the 16S rRNA gene. However, the species delineation relies on the base sequence of the 16S rRNA gene is inadequate to characterize a single identical species . The 16S rRNA base sequence that shows low similarity cannot yet provide a complete taxon description (Sune et al., 2020). When the similarity presentation is ≥ 99%, it suggests that the species being compared are identical. When it falls between 97-99%, it implies that the isolates being compared belong to different species within the same genus. If the presentation is <97%, it suggests that the isolate might qualify as a new bacterium (Rahardiyan & Moko, 2023).

**Relationships between *Catharatus roseus* Endophytic Bacteria**

The familial connections among endophytic bacteria within the isolates under investigation can be ascertained through a phylogenetic tree, which examines genetic distances relative to several previously discovered bacteria (Sadiqi et al., 2022) . Phylogenetics is most often used to see the diversity of living things through the reconstruction of kinship relationships (Szymczak et al., 2020). In this study, the statistical robustness of the phylogenetic tree was assessed by employing the bootstrap method with 500 repetitions, followed by the construction of a new phylogenetic tree based on the results. Figure 6 illustrates the phylogenetic tree derived from the analysis of the 16S rRNA.

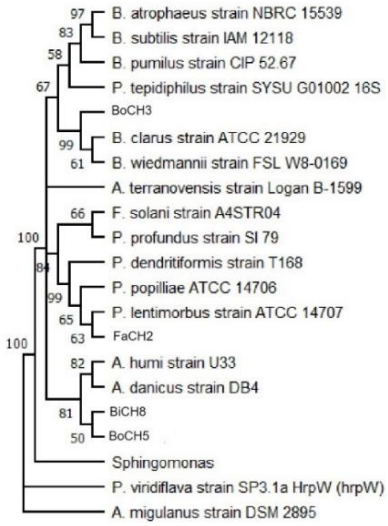


**Figure 6**. Phylogenetic tree of endophytic bacteria *C. roseus* from Timor, East Nusa Tenggara and several endophytic bacteria discovered previously. Figures indicate bootstrap values

Figure 6 shows that the genetic distance between endophytic bacterial isolates can be analyzed by branch or horizontal chain length according to the shown scale. The results showed that bacterial isolates were in three groups, namely the genus *Bacilae*, *Paenibacillae* and *Aneurinibacillae*. Bacteria of the genus *Paenibacillus*is are those that can promote plant growth directly through biological nitrogen fixation, and can provide protection against herbivorous insects and plants because this bacterium can produce various insecticides and antimicrobials by triggering a hypersensitive plant defense response known as induced systemic resistance (ISR) (Jiang et al., 2022). These antimicrobial derivatives also have applications in treatment including polymixy. *Bacillus wiedmannii* is a gram-positive bacterium that is long rod-shaped, having ellipsoidal endospores, is positive for egg yolk lecithinase as well as having the ability to hydrolyze casein and starch, and has the ability to produce HBL toxin and non-hemolytic toxin NHE, producing antimicrobial compounds against *S. aureus* bacteria and contains anticancer compounds against the growth of Hela cells. On the other hand, *Aneurinibacillus humi* is a bacterium belonging to the genus *Aneurinibacillus*, characterized by being gram-positive, spore-forming, aerobic, and motile (Miller et al., 2016).

Genetic distance analysis between endophytic bacterial isolates showed that FaCH2 isolate had a similarity of 63% and the closest genetic distance was 0.000 with *Paenibacillus lentimorbus* strain ATCC 14707, BoCH3 isolate has 99% similarity and genetic distance of 0.003 with *Bacillusclarus* ATCC 21929 and *Bacillus strains wiedmannii* strain FSL W8-0169, isolates BoCH5 and BiCH8 have a similarity of 81% and a genetic distance of 0.033 with *Aneurinibacillu humi* strains U33 and *Aneurinibacillu danicus* strain DB4 (table 4).

**Table. 4.** Analysis of genetic distance between species from the 16S rRNA coding gene

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. P._dendritiformis_strain_T168 | | | | | | | | | | | | | | | | | | | | | |
| 2. A._migulanus_strain_DSM_2895 | 0.668 | | | | | | | | | | | | | | | | | | | | |
| 3. P._tepidiphilus_strain_SYSU_G01002 | 0.078 | 0.639 | | | | | | | | | | | | | | | | | | | |
| 4. B._clarus_strain_ATCC_21929 | 0.075 | 0.657 | 0.048 | | | | | | | | | | | | | | | | | | |
| 5. A._humi_strain_U33 | 0.069 | 0.651 | 0.048 | 0.075 | | | | | | | | | | | | | | | | | |
| 6. B._wiedmannii_strain_FSL_W8-0169 | 0.075 | 0.657 | 0.048 | 0.000 | 0.075 | | | | | | | | | | | | | | | | |
| 7. F._solani_strain_A4STR04 | 0.042 | 0.657 | 0.057 | 0.072 | 0.051 | 0.072 | | | | | | | | | | | | | | | |
| 8. P._profundus_strain_Sl_79 | 0.036 | 0.651 | 0.048 | 0.069 | 0.051 | 0.069 | 0.027 | | | | | | | | | | | | | | |
| 9. B._pumilus_strain_CIP_52.67 | 0.066 | 0.645 | 0.030 | 0.045 | 0.042 | 0.045 | 0.045 | 0.042 | | | | | | | | | | | | | |
| 10. P._lentimorbus_strain_ATCC_14707 | 0.003 | 0.666 | 0.075 | 0.072 | 0.066 | 0.072 | 0.039 | 0.033 | 0.063 | | | | | | | | | | | | |
| 11. B._atrophaeus_strain_NBRC_15539 | 0.066 | 0.648 | 0.039 | 0.042 | 0.054 | 0.042 | 0.057 | 0.051 | 0.021 | 0.063 | | | | | | | | | | | |
| 12. B._subtilis_strain_IAM_12118 | 0.063 | 0.648 | 0.036 | 0.039 | 0.051 | 0.039 | 0.057 | 0.048 | 0.018 | 0.060 | 0.006 | | | | | | | | | | |
| 13. A._terranovensis_strain_Logan | 0.075 | 0.663 | 0.066 | 0.075 | 0.066 | 0.075 | 0.072 | 0.066 | 0.063 | 0.072 | 0.048 | 0.051 | | | | | | | | | |
| 14. P._popilliae_ATCC_14706 | 0.006 | 0.663 | 0.072 | 0.069 | 0.063 | 0.069 | 0.036 | 0.030 | 0.060 | 0.003 | 0.060 | 0.057 | 0.075 | | | | | | | | |
| 15. A._danicus_strain_DB4 | 0.075 | 0.645 | 0.051 | 0.078 | 0.021 | 0.078 | 0.060 | 0.057 | 0.054 | 0.072 | 0.063 | 0.060 | 0.063 | 0.075 | | | | | | | |
| 16. K4_DAK | 0.096 | 0.651 | 0.066 | 0.090 | 0.033 | 0.090 | 0.069 | 0.069 | 0.060 | 0.093 | 0.063 | 0.066 | 0.075 | 0.090 | 0.042 | | | | | | |
| 17. K3_DPAP | 0.102 | 0.642 | 0.072 | 0.096 | 0.039 | 0.096 | 0.075 | 0.075 | 0.066 | 0.099 | 0.069 | 0.072 | 0.081 | 0.096 | 0.048 | 0.036 | | | | | |
| 18. K2_dp2K | 0.072 | 0.657 | 0.045 | 0.003 | 0.072 | 0.003 | 0.069 | 0.066 | 0.042 | 0.069 | 0.039 | 0.036 | 0.072 | 0.066 | 0.075 | 0.087 | 0.093 | | | | |
| 19. K1_dp3 | 0.003 | 0.666 | 0.075 | 0.072 | 0.066 | 0.072 | 0.039 | 0.033 | 0.063 | 0.000 | 0.063 | 0.060 | 0.072 | 0.003 | 0.072 | 0.093 | 0.099 | 0.069 | | | |
| 20. Sphingomonas | 0.163 | 0.636 | 0.066 | 0.063 | 0.078 | 0.063 | 0.054 | 0.069 | 0.063 | 0.066 | 0.072 | 0.072 | 0.072 | 0.069 | 0.081 | 0.093 | 0.096 | 0.060 | 0.166 | | |
| 21. P._viridiflava_strain_SP3.1a_HrpW | 0.057 | 0.651 | 0.054 | 0.069 | 0.057 | 0.069 | 0.066 | 0.054 | 0.057 | 0.054 | 0.063 | 0.063 | 0.063 | 0557 | 0.063 | 0.060 | 0.069 | 0.066 | 0.054 | 0.599 | |

This research is the first study on the isolation and characterization of endophytic bacteria from C. roseus native to Timor Island, East Nusa Tenggara. The species of endophytic bacteria from Timor Island have unique characteristics different from those found in other regions. The antibacterial potential of endophytic bacteria against MRSA is a new approach. MRSA is a highly resistant pathogen and difficult to treat with conventional antibiotics, so discovering new antibacterial sources from endophytes is of significant value. This research has the potential to produce new bioactive compounds generated by endophytic bacteria that have not been reported before. These compounds could open opportunities for the development of new antibiotics.

## CONCLUSION

Based on the research findings, it can be concluded that four isolates of endophytic bacteria from the Catharanthus roseus plant originating from Timor, East Nusa Tenggara, were found to have high potential as producers of antibacterial agents against Methicillin-Resistant Staphylococcus aureus, namely isolates FaCH2, BoCH3, BoCH5, and BiCH8. The four isolates of endophytic bacteria from C. roseus have morphological similarities with Bacillus sp and genetic similarities with Paenibacillus dendritiformis at 91.81%, Bacillus cereus at 92.65%, Aneurinibacillus migulanus at 84.76%, and 90.65%. The endophytic bacterial species identified through 16S rRNA gene analysis have the potential to be classified as novel bacterial species capable of producing new antibacterial agents, given the similarity level is below 97%.

Further research should focus on the identification and characterization of the bioactive compounds produced by the endophytic bacteria. More in-depth chemical analyses, such as chromatography and mass spectrometry, can be used to determine the structure of the compounds from the endophytic bacteria found in this study.

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