Stability of Production of Active Antibiotic Compounds by Andalas Endophite Bacteria (*Morus Macroura* Miq.) at Several Subculture Frequency

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Abstract. To maintain the viability of bacteria during the development and utilization process, it is necessary to rejuvenate the bacteria through a subculture process. It is interesting to know whether the activity of the antibiotic compounds and the genetic stability of Andalas endophytic bacteria will remain the same when the bacteria are subcultured repeatedly on the growth medium. The purpose of this study was to determine the stability of production of antibiotic active compounds and genetic stability by Andalas endophytic bacteria at several subculture frequencies. The subculture of Andalas endophytic bacteria in this study was carried out on B.J.T.A.2.1 isolates, starting from the 3rd passage (P.3) to the 25th passage (P.25). Subcultures were performed on triple NA slanted agar medium. Stability of production of active antibiotic compounds was carried out using the diffusion method with the point inoculation technique. The genetic stability test of Andalas endophytic bacteria was carried out by comparing the isolate's 16S rRNA sequence (P.3) with (P.25). The results showed that the stability of the production of antibiotic active compounds decreased after repeated subcultures in the 7th passage. The results of genetic stability research by Andalas endophytic bacteria isolate B.J.T.A.2.1 at several subculture frequencies found 3 nucleotide variations of the 16S rRNA gene between the two Andalas endophytic bacteria isolates.

Keywords: Andalas endophytic bacteria · subculture (serial passage) · stability

1 Introduction

Infectious diseases have caused the death of approximately 13 million people in the world every year, especially in developing countries such as Indonesia. The use of antibiotics is an effort to control infectious diseases. In recent years there has been an increase in the rate of resistance to antibiotics [1]. Based on data from the World Health Organization

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(WHO) [2], it was found that Staphylococcus sp. Resistance to the antibiotic methicillin was around 63% in 2009. This percentage increased to 80% in 2013.

According to Kuntari [3], the discovery and development of new antibiotic compounds will affect the treatment of infectious diseases and improve the quality of human life. Therefore, exploration is needed to find new sources of antibiotics. One of them is by utilizing endophytic bacteria.

Endophytic bacteria are bacteria that live in the tissues of the host plant without causing disease symptoms. These bacteria do not harm their host, even in a mutualistic symbiosis to obtain nutrients from plant metabolism [4]. One of the medicinal plants that can be used as a source of endophytic bacteria producing antibiotic compounds is the Andalas plant (*Morus macroura* Miq.). Andalas plants contain several phenolic compounds such as morasin B, morasin P, mulberoside C, mulberofuran, stilbene derivatives, coumarin, umbeliferone, 2-arylbenzofuran, moracin M and β-resolsiladehid. These compounds are active ingredients that have antibiotic activity [5].

Research conducted by Afifah [6] succeeded in isolating 11 isolates of endophytic bacteria from Andalas plant stems, of which 10 isolates had the ability to produce antimicrobial compounds. Furthermore, research conducted by Putri [7] succeeded in isolating endophytic bacteria from the leaves of the Andalas plant. A total of 4 isolates of endophytic bacteria from Andalas leaves have the potential to produce antimicrobial compounds for gram-positive bacteria (*S. aureus)* and fungi (*C. albicans).*

The development of active antibiotic compounds from bacteria requires a long process in order to be produced on an industrial scale. Optimum production conditions require extensive optimization processes and take a long time. To maintain the viability of bacteria during the development and utilization process, it is necessary to rejuvenate the bacteria through a subculture process. Subculture is the process of transferring microbes from one growth medium to a new medium to allow microbes to grow properly. The frequency of subcultures performed is often referred to as serial passage [8].

Bacteria can experience changes in their ability to produce antibiotic compounds due to processing or storage, including the subculture process carried out. According to Torres [9], the frequency of *M. tuberculosis* subcultures observed every week for 6 months in liquid media can cause bacteria to be resistant to fingerprint-modified drugs. The effect of this subculture frequency will produce mutations that affect genetic characteristics and virulence. The effect of subculture frequency can also be observed physiologically. Research conducted by Leeds [10] showed that the frequency of subcultures (10 times) could change the genetics of *C. difficile* clones, which in turn affected the expression of these clones. This genetic change occurs due to the accumulation of mutations in each subculture process carried out.

It is interesting to know whether the activity of the antibiotic compounds and the genetic stability of Andalas endophytic bacteria will remain the same when the bacteria are subcultured repeatedly on the growth medium. The activity of antibiotic compounds can be measured by the disc diffusion method. The principle of this method is based on the ability of the tested active compounds to diffuse in the agar medium and inhibit the growth of the test microbes [11]. Furthermore, the genetic stability of endophytic bacteria can be observed from the 16S rRNA gene. The 16S rRNA gene is one of the genes that has been well characterized so that it is often used as the basis for identification

of microorganisms. The 16S rRNA gene has a nucleotide of 1500 bp consisting of conservative and varied regions. A varied base sequence can be used to track diversity and determine the formation of new strains within a species [12].

2 Methods

2.1 Andalas Endophytic Bacteria Isolate B.J.T.A.2.1

Endophytic bacteria Andalas B.J.T.A 2.1 is a research laboratory stock in the Department of Biology, Faculty of Mathematics and Natural Sciences, UNP. This bacterium was isolated by Afifah [6] from Andalas stems. Bacterial stock is rejuvenated by growing bacteria on a nutrient agar growth medium. Bacterial cultures were incubated in an incubator at 37ºC for 24 h.

2.2 Microbial Strains

Following standard bacterial strains were used in this study belonging to Gram positive and Gram negative species: *Staphylococcus aureus* (ATCC.25923) and *Escherichia coli* (ATCC.EC 25922). They were obtained from mikrobiology laboratotium, Medical Faculty, University of North Sumatra.

2.3 Stability Test of Subcultured Antibiotic Active Compound Production

Antibiotic activity test was carried out at each passage frequency (starting from (P.3)). Previously, the test microbe whose turbidity had been equalized to the McFarland's 0.5 scale was applied evenly to the NA medium using a sterilized cotton swab (Whitman, 2004). The isolate of B.J.T.A.2.1 (P.3) to be tested, was taken one ose of oblique culture culture and placed using a sterile toothpick on the medium that had been inoculated with the test microbe. Furthermore, the petri dish was incubated at room temperature with the condition of the petri dish inverted. The diameter of the clear zone around the inoculation point was measured after being incubated for ± 24 h.

2.4 16S rRNA Gene Sequencing

In this study, the genetic stability of the isolate B.J.T.A.2.1 was also observed, by comparing the 16S rRNA sequence of the isolate (P.3) with (P.25). The DNA isolation, PCR and sequencing processes for these two isolates were carried out separately.

2.4.1 Genomic DNA Isolation

Isolation of the bacterial genome was carried out by the boilling method (Dashti, 2009). A total of 2–3 oses of bacterial colonies were put into a 1.5 ml Eppendorf tube containing 200 l of 1/10 TE buffer pH 8. The suspension was vortexed until homogeneous. The boiling process was carried out in a water bath at a temperature of 95-100oC, for 30 min. The suspension was then centrifuged at 12,000 rpm for 15 min to separate the supernatant and pellet. The supernatant was transferred to a new Eppendorf tube, and stored at -20ºC until use.

2.4.2 PCR and Electrophoresis of 16S rRNA Gene of Andalas Plant Endophytic Bacteria

The bacterial 16S rRNA gene was amplified from the bacterial genome, which had been previously isolated, using universal primers 27F (forward) and 1492R (reverse). PCR reactions were carried out in a 25 l reaction, consisting of 6.5 l ddH2O, 12.5 l Kit Dream Taq master mix PCR (Thermo Scientific), 2 l each of the forward and reverse primers, and 2 l of template DNA. The temperature used was initial denaturation of 95ºC for 3 min, followed by 35 cycles consisting of denaturation of 95ºC for 45 s, annealing at 55ºC for 30 s, and elongation at 72ºC for 2 min. The reaction was closed by final elongation at 72ºC for 7 min. Analysis of PCR products was carried out by electrophoresis technique using 1% agarose gel. The results of the electrophoresis were then checked with an electrophoresis doc gel.

2.4.3 Effect of Particle Size

The PCR results were sequenced to determine the nucleotide sequence. Sequencing was carried out at Macrogen Singapore with an automated DNA sequencer (ABI Prism 3100 Genetic Analyzer, Applied Biosystem, USA). Each fragment of the PCR results was sequenced twice each, using internal forward and reverse primers. The results of the sequencing were analyzed using BioEdit software and then assembled into one complete nucleotide sequence of the 16S rRNA gene. Alignment was carried out by comparing the results of the sequencing with the standard sequence (ie the same bacterial 16S rRNA gene obtained from NCBI). Genetic stability was determined by performing pairwise alignment analysis with Bioedit software. Alignment results were used to determine the percentage of homology between the two isolates.

3 Result and Discussion

3.1 Stability Test for Production of Antibiotic Active Compounds at Several Subculture Frequency

The results of the stability test of the production of antibiotic active compounds at several subculture frequencies showed that in general there was a decrease in antibiotic activity by endophytic bacteria at several subculture frequencies during the observation time. It can be seen in Fig. 1 that until the 7th passage there was a significant decrease in the production of antibiotic active compounds by Andalas endophytic bacteria. After the 7th to the 25th passage, the productivity of antibiotic compounds tends to be constant.

The process of subculture of Andalas endophytic bacteria repeatedly will result in a decrease in the production of antibiotic compound activity. According to Marsono [13], the decrease in the inhibitory power of bacteria is due to the decrease in the content of the active substance or active compounds contained during the storage and processing process. Furthermore, according to Yun [14], the stability of the synthesis of bacterial metabolic products is influenced by several factors such as the stability of the active ingredient, the interaction between the active ingredient and the test bacteria, the environmental conditions experienced during the storage and use process, and environmental factors.

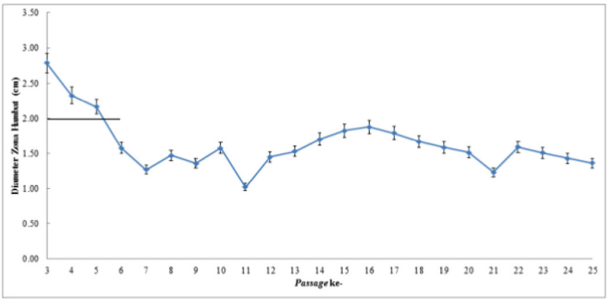

Fig. 1. Stability of Antibiotic Active Compounds of Andalas Endophytic Bacteria in Passage-3 to Passage-25 Subcultures

In terms of antibiotic activity produced by endophytic bacteria, the decrease in the production of antibiotic active compounds by endophytic bacteria may be due to the adaptation process of bacteria to the new growth medium. As stated by Strobel [15], endophytic bacteria are bacteria that live and associate with plant tissue without causing any disease symptoms in the plant. The presence of endophytic bacteria in plant tissue plays a role in increasing the plant's defense system against disease because it has the ability to produce antibiotic compounds, enzymes, salicylic acid, ethylene and other secondary compounds that play a role in inducing plant resistance.

The interaction between endophytic bacteria and their host in producing antibiotic compounds was proven by Das [16]. Where the production of antibiotic active compounds by endophytic bacteria can be influenced by the addition of plant extracts of origin into the fermentation medium. The results showed that there was an increase in the activity of producing active compounds in the medium added with plant extracts compared to bacteria which were only grown on Nutrient Broth (NB) synthetic medium. This study shows that there are certain components in the original plant extracts that induce the ability of endophytic bacteria to produce antibiotic active compounds.

3.2 Genetic Stability Test at Multiple Subculture Frequency

Genetic stability test was carried out by comparing the 16S rRNA gene sequences of Andalas endophytic bacteria passage 3 with passage 25. For sequencing, DNA templates are needed which are synthesized through PCR reactions. From the electrophoresis results of the PCR product in Fig. 2. Successfully amplified the 16S rRNA gene from Andalas endophytic bacteria isolate B.J.T.A.2.1. The DNA band was between the HindIII marker band, measuring 564 bp and 2027 bp. Based on the position of the forward and reverse primers, the magnitude of the PCR product was about ± 1500 bp.

PCR products are used as templates in the sequencing process. The length of the 16S rRNA gene sequence that can be analyzed properly is about 1436 bp. The results of 16S rRNA gene sequencing of Andalas endophytic bacteria in this study found nucleotide variations in passage 3 and passage 25. Based on the pairwise alignment analysis of the sequences obtained, the homology value is 99.7%. According to Yang and Bruce [17], pairwise alignment is a method that can be used to align two sequences, so that similarities (homology between the sequences) can be seen.

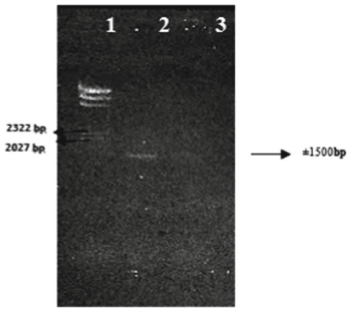

Fig. 2. Electrophoresis Results of PCR Products for Andalas 16S rRNA Endophytic Bacteria: (1) HindIII marker. (2) Isolate B.J.T.A.2.1 3rd Passage. (3) Isolate B.J.T.A.2.1 Passage

Table 1. Nucleotide Variation of the 16S rRNA Gene of Andalas Endophytic Bacteria between B.J.T.A.2.1 Isolate 3rd Passage and 25th Passage

Position (bp)	Nucleotide Variation	
	BJTA.21 (pass-3)	BJTA.21 (pass-25)
843	G	C
1403	G	C
1442	G	C

The nucleotide variations found in this study were quite convincing because based on observations on the chromatograms of the sequencing results, only one peak was found in the varying regions. According to Bangol et al. [18], good sequencing results are shown by chromatograms with high peaks and are separated from each other. Poor sequencing results are indicated by sloping peaks and overlapping or there are double peaks on the chromatogram.

Based on the results of the pairwise alignment, it was also known that there was a variation in the nucleotide of the 16S rRNA gene between the two isolates of Andalas endophytic bacteria. The types of nucleotide variations and chromatogram descriptions resulting from the sequencing process can be seen in Table 1.

Based on the data in Table 1, it can be seen that there were 3 nucleotide variations of the 16S rRNA gene between the two Andalas endophytic bacteria isolates. Variations occurred at the positions of 843 bp, 1403 bp and 1441 bp. According to Clarridge [12], the 16S rRNA gene consists of conservative and varied regions. Variable base sequences can be used to track diversity and determine the formation of new strains within a species. The analysis carried out by Chakravorty et al., [19] mapped the varied regions of the 16S rRNA gene, the results found 9 regions that had high variation, namely: at gene sequences 69–99, 137–242, 433–497, 576- 682, 822–879, 986–1043, 1117–1173, 1243–1294 and 1401–1465. Based on the results of the study, it can be seen that all variations occur in areas that do have a tendency to vary (areas to 433–497 and 1401–1465).

The nucleotide changes that occur are the same, namely guanine (G) turns into cytosine (C). This change can also be referred to as a mutation process. Mutation is a change in the nucleotide sequence of the DNA sequence of an organism that results in genetic diversity. Mutations occur randomly, and spontaneously. Mutations occur when there is an error in the DNA replication process that the DNA repair system escapes to repair. In this study, the mutations that occur are substitution mutations.

Changes in the nature and genetics of bacteria due to subculture processes (especially in the use of biotechnology) need attention, because they can affect the expected results. Based on the information obtained from the ATCC® bacteria culture guide, it is known that ideally the maximum number of subcultures that can be performed on the reference bacteria is no more than 5–10 times. The number of subcultures depends on the type of bacteria and the medium used. To increase the number of subcultures of endophytic bacteria, it is necessary to optimize the growth medium that is made similar to the original host of the bacteria.

In this study, subcultures of Andalas endophytic bacteria up to passage 25 were still able to produce antibiotic compounds. For long-term use, it is necessary to observe a larger number of subcultures. Thus, the activity profile of the production of antibiotic compounds by Andalas endophytic bacteria can be well understood.

4 Conclusion

The production stability of the active compounds of Andalas endophytic bacteria decreased during the subculture process until the 7th passage, and the subsequent subculture process until the 25th passage. From the results of gene sequencing, three nucleotide variations were found in the 16S rRNA gene of Andalas endophytic bacteria, passage 3 and passage 25.

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