Screening of Antibacterial Potency and Molecular Identification of Endophytic Bacteria from Soursop Leaf (*Annona muricata* L.)

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Abstract: Soursop leaves (*Annona muricata* L.) is one of the medicinal plants identified as a source of endophytic bacteria producing secondary metabolites. Several studies have reported that secondary metabolite compounds extracted from soursop leaves have inhibitory activity against pathogenic bacteria and fungi and have anticancer activity. This study aims to isolate endophytic bacteria of soursop leaf and identify molecular isolates producing antibacterial metabolites by PCR method. This study began with endophytic bacterial isolation of soursop leaves, followed by screening for antibacterial potency using disc diffusion method and identification of molecular isolates which had the highest antibacterial activity. After isolation, three isolates were obtained: BW-1LM, BW-2LP, and BW-3LK. The result of antibacterial activity test showed that BW-1LM isolate had the highest activity against bacterium of *Bacillus subtilis* and *Salmonella typhi* test. Molecular identification was obtained by BW-1LW isolate having 99% similarity level to *Bacillus licheniformis* DSM 13 strain. The conclusion is soursop leaves contains endophytic bacteria which have antibacterial activity against *Bacillus subtilis* and *Salmonella typhi*.

1 INTRODUCTION

Indonesia is a country rich in natural resources and one of them is medicinal plants. The different types of plants that exist are crucial natural resources in producing various bioactive compounds that are potential to be developed. The plants produce secondary metabolites with molecular structures and diverse biological activities, and has good potential to be developed into a cure for various diseases (Radji, 2005). The resulting secondary metabolites are thought to be the result of coevolution or genetic transfer of host plants into endophytic microbes (Tan and Zou, 2001).

Endophytic microbes are microorganisms whose habitat is within the plant organs over a period and can produce secondary metabolites that have bioactivity, such as enzymes, antimicrobial agents, anti-fungus, anticancer, and plant growth regulators (Kumala, 2014). One of the medicinal plants that can be used as a source of endophytic bacteria is soursop leaf (*Annona muricata* L.). Soursop is a plant that has medical properties and is widely used in Indonesia. The part of soursop plants that are widely used for medicinal purpose is the leaves. Soursop leaf contains saponin compounds, flavonoids, coumarins, alkaloids, and tannins. Haro *et al*. (2014) reported soursop leaf extract methanol might inhibit *Escherichia coli* and *Staphylococcus aureus* bacteria. The information about the ability of endophytic bacteria in producing bioactivity compounds encourage the research on endophytic bacteria in soursop leaf.

The polymerase chain reaction known as Polymerase Chain Reaction (PCR) is an enzymatic synthesis process to amplify nucleotides in vitro (Fatchiyah, 2011). The PCR process is a recurrent cycle process, including denaturation, annealing, and extension by DNA polymerase enzymes. The amplification process was continued by the analysis of electrophoresis and identification using the marker gene of 16S rRNA. Genes 16S are genes specific to prokaryotic species (Clarridge, 2004). Resti *et al*. (2013) reported results of endophytic bacteria from onion plants obtained 82 isolates endophytic bacteria, and six isolates of them have potential ability to bacterial leaf blight disease.

This study is conducted to obtain endophytic bacteria from soursop leaves that have antibacterial activity and molecularly identifies endophytic

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bacteria of soursop leaf (*Annona muricata* L.) producer of antibacterial secondary metabolites. The antibacterial activity test was performed by disc diffusion method. Isolates that had the greatest antibacterial activity were continued by identifying endophytic bacteria molecularly using 16S rRNA gene.

2 MATERIALS AND METHODS

2.1 Materials

Microcentrifuge refrigerator (PerfectSpin 24 Plus), PCR thermo cycler (Tanach RAY-MG48), electrophoresis (Mupid EXU), UV transilluminator (Genesys 20), Rotary shaker (Eyela). Soursop leaf samples (*Annona muricata* L.) NaOCl 5.3%, medium Nutrient Agar (NA) and Nutrient Broth medium (NB), EDTA, Lysozyme, Isopropanol, Ethanol 70%, and Wizard Genomic DNA Purification Kit from Promega, Forward primary 27f and reverse primary 1492r (Rosita 2012). Nuclease-free water, GoTaq Green PCR Master Mix of Promega, Gel agarose, ethidium bromide (EtBr), Tris Acetate EDTA (TAE) buffer solution, 1kb ladder DNA (Promega) and loading dyes (Thermo Scientific), Medium Muller Hinton Agar MHA), bacterial cultures of *Bacillus subtilis* and *Salmonella typhi* test.

2.2 Sample Preparation and Plant Identification

Plant samples were taken from Tangerang area and then identified in Herbarium Bogoriense, Botanical Field of LIPI Research Center, Cibinong-Bogor.

2.3 Isolation of Endophytic Bacteria

This stage applied the method used by Kumala (2014) and began with samples of fresh soursop leaves. The leaves were washed with water and then cut. The sample pieces were then sterilized by being immersed in 75% ethanol for 1 minute and then soaked with 5.3% sodium hypochlorite for 5 minutes. Then the sample was rinsed three times with 75% ethanol. The sterile samples were then grown on Nutrient Agar (NA) media which had been added with nystatin and incubated in dark spaces at room temperature and observed until growing colony appeared (Pratiwi, 2008).

2.4 Characterization and Gram Staining

This study used microscopic and macroscopic observations. Macroscopic observations include colony pigmentation, colony form, colony elevation, colony surface, and colony consistency. Microscopic observations include the shape and colour of cells using Gram staining.

Gram staining begins by doing the bacterial scraping on the glass object then sprayed with crystals violet for 1 minute as much as one drop, then washed with running water and dried again. After that, one drop of Lugol solution is added and let stand for 1 minute, then washed with water and dried again. As decolorizing agent, drops of 96% alcohol is added to the object glass until the dye is faded, and then rinsed with water and allowed to dry. The last stage, one drop of safranin is given as a counterstain, and let stand for 30 seconds (Pratiwi, 2008).

2.5 Fermentation of Endophytic Bacteria

The pure cultured bacteria obtained are inoculated in a test tube containing medium nutrient broth (NB), then incubated for 7x24 hours with a shaker speed of 145 rpm. After that, every 1x24 hours as much as 1 ml of the mixture was taken and centrifuged for 3 minutes at a speed of 5000 rpm. The centrifugation supernatant was transferred to a new micro tube and stored at 40°C. (Kumala *et al*., 2007)

2.6 Screening Antibacterial Activity of Endophytic Bacterial

Screening by disc diffusion method used *Salmonella typhi* and *Bacillus subtilis*. The endophytic bacterial isolates from tilted agar were regenerated into nutrient agar medium (NA), whereas the test bacteria were regenerated into five mL nutrient broth medium (NB). Suspension of test bacteria that has been fulfilled the transmittance value of 25% is taken as much as 10% liquid culture of the test bacteria is fed into MHA media with a temperature of 37 ℃.

Paper discs that have been soaked fermented supernatant then placed on a medium that has been inoculated pathogenic bacteria. Subsequently incubated for 24-48 hours at 37 °C and was observed antibacterial activity with the presence of an obstacle zone around the disc paper (Simarmata *et al*., 2007).

2.7 Isolation of Genomic DNA Bacterial Endophytes

The bacterial isolates to be used for the DNA isolation process were first cultured in Nutrient Broth medium (NB) and incubated at 37 °C for 24 hours. The isolation process is based on the protocol contained in the Genomic DNA Purification Kit (Promega, 2014). A total of 1.5 ml bacterial culture was transferred into a sterile 2.0 ml micro centrifuge (microtube) and centrifuged at 13000 times for 2 min. The forming supernatant was discarded, then resuspended complete bacterial cell deposits with 480 µl EDTA 50Mm. A further 120 µl of lysozyme was homogenized and incubated at 37 °C for 30 minutes, centrifuged for 2 min at a rate of 13000 times and disposed of the supernatant formed. The next step was added 600 µl nuclei lysis solution then homogenized and incubated at 80 °C for 5 minutes to lyse the cells, the sample was allowed at room temperature then added three µl RNase solution then homogenized by reversing the tube. After that, it was incubated at 37 °C for 30 minutes. The next step was added 200 µl protein precipitation solution then homogenized for 20 seconds with high vortex speed, then incubated sample at the cold temperature for 5 minutes and centrifuged for 3 minutes at 13000 times.

The supernatant containing the DNA is further incorporated into a new microcentrifuge tube containing 600 µl of isopropanol, homogenized gently until visible strands of DNA thread. After that, centrifuged at a rate of 13000 times for 2 minutes, then the supernatant formed was carefully removed, and the tube was dried with absorbant paper. A total of 600 µl of 70% ethanol was added to the tube containing the DNA, homogenized the tube gently to wash the DNA pellets. After that, it was centrifuged at 13000 times for 2 minutes, and the tube was dried for 15 minutes. The last stage was added 100 µl DNA rehydration solution then incubated at 65 °C for 1 hour. DNA was stored at 2-8 °C (Promega, 2014).

2.8 DNA Amplification With PCR

The bacterial genomic amplification process was based on the protocol contained in the Maxima Hot Start Green PCR Master Mix (2X), using primer 27f and primer 1492r. A total of 25 µl Maxima Hot Start PCR master mix (2X) was inserted into a 0.5 ml microtube. Nuclease-free water was added as much as 9 µl, then the mixture was resolved until dissolved completely by homogenized. Furthermore, homogeneous mixtures were added by primer 27f and primary 1492r, 2.5 µl of each. Then, it was added with 3 µl of DNA and homogenized. The amplification process was performed using Bartlett and Stiling (2003) method, namely: Initial denaturation at 95ºC for 5 minutes for 1 cycle, Denaturation at 95°C for 1 minute for 30 cycles, Annealing at 56ºC for 1 minute for 30 cycles, Extension at 72ºC for 1 minute for 30 cycles, Final extension at 72ºC for 10 minutes for 1 cycle. The obtained amplicon was observed with electrophoresis using a 0.8% agarose gel (Bartlett and Stiling, 2003).

2.9 Sequencing of Gen 16S rRNA

The sample was put into a 0.2 ml dry and sterile micro tube, then sent to Eijkman Molecular Biology Institute, Jakarta Indonesia for further purification and sequencing.

2. 10 Identification of 16S rRNA Bacterial Endophytic Gene

DNA sequenced were analyzed with the bioedit program and then compared with the sequence database at the nBLAST site (http:/www.blast.ncbi.nlm.nih.gov/). The result obtained in this research was then compared to the data on Gene Bank.

3 RESULTS AND DISCUSSION

3.1 Isolation and Characterization of Endophytic Bacterial Morphology

The endophytic bacteria may be associated with the host plant, the function is to help the metabolism process of the host plant and to produce secondary metabolites that are similar to the host plant compounds (Kumala, 2014). Isolation of endophytic bacteria was done by direct planting method on Nutrient Agar (NA) medium. From the isolation process, we obtained three endophytic bacterial isolates which are labelled as BW-1LM, BW-2LP, and BW-3LK. The macroscopic observation results indicated that BW-1LM isolate is yellowish white, irregularly shaped and has mucous consistency. While microscopic results showed that bacterial cells are identic with the *Bacillus* genus included in Gram-positive bacteria. Macroscopic observation of 2LP isolate described that this isolate is BW-white,

Table 1: Characteristics of endophytic bacterial isolates from soursop leaf (*Annona muricata* L.).

Isolate Code	Morphology						
	Morphology	Pigmentation	Consistency	Elevation	Edge	Cell Shape	Color
BW-1 LM	Irregular	White Yellowish	Slimy	Arise	Irregular	Basil	Purple
BW-2 LP	Irregular	White	Slimy	Arise	Irregular	Circular	Purple
BW-3 LK	Irregular	White	Slimy	Arise	Irregular	Circular	Red

irregularly shaped and have slimy consistency, while the results of microscopic bacteria cells are round and a Gram-positive-bacteria. The macroscopic observations of BW-3LK isolate are white, irregularly shaped and slimy. Microscopic observations of BW-3LK isolate showed that bacterial cells are spherical and included in Gram negative bacteria. Those observation results of the endophytic bacteria characteristics can be seen in the Table 1.

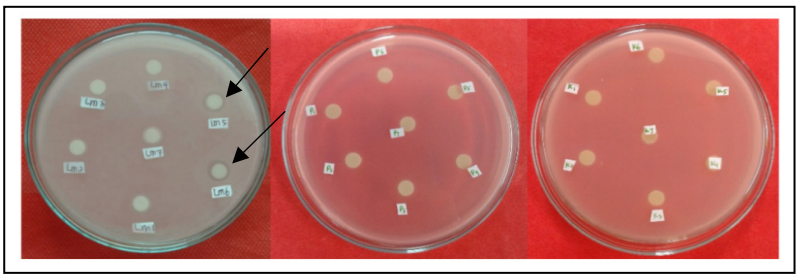
In Table 1, it can be seen that all of three isolates have different characteristics. From the Gram staining result, there are red colored bacteria that have been known as Gram-negative bacteria, while the purple colored bacteria are Gram-positive bacteria (Radji, 2010). Gram staining can also show the presence or absence of peptidoglycan content in bacterial cell membranes. If the bacterial cell membrane does not have a peptidoglycan compound, the appearance of the resulting color after being observed under a microscope is red (Pratiwi, 2008).

3.2 Screening for Antibacterial Activity

The antibacterial activity test was performed by inoculating the discs that had been immersed in the supernatant of endophytic bacterial fermentation of soursop leaf on Muller Hinton Agar (MHA) medium. Those media were mixed with *Bacillus subtilis* isolates (Gram-positive) and *Salmonella typhi* isolates (Gram-negative). Three endophytic bacterial isolates were inoculated on NB fermentation medium, then fermented for 7x24 hours at 145 rpm. From the test results, only BW-1 LW isolate showed antibacterial activity against both test bacteria.

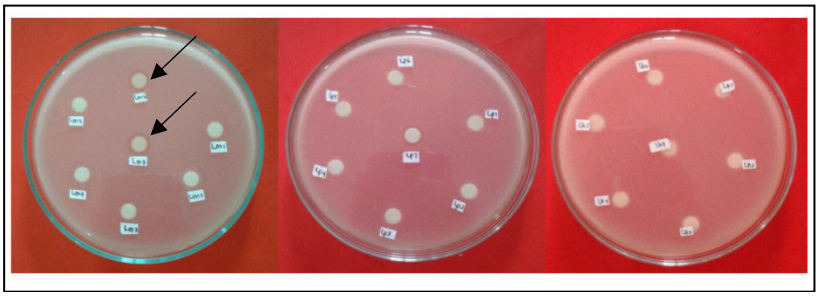
The results of the activity test on *B. subtilis* bacteria can be seen in Figure 1. Based on this image, the inhibition zone can be seen around the disc paper on fermentation products on the 5th and 6th days. Figure 2 is the result of the endophytic bacteria secondary metabolite activity test against *S. typhi* bacteria, on the 6th and 7th day of fermentation products. There was an inhibition zone around the disc paper, while the other two isolates were unable to inhibit the growth of the two test bacteria.

Table 2 shows the inhibitory zone diameter of bacterial isolate BW-1 LM. This table shows the activity produced by BW-1 LM isolate on 5th, 6th, 7th day fermentation. From the data, it can be seen that BW-1LM endophytic bacterial isolate has antibacterial activity against both test bacteria on 6th-day fermentation.



BW-1LM BW-2LP BW-3LK

Figure 1: Secondary Antibacterial Activity Test Results of Endophytic Bacteria from Soursop Leaves (*Annona muricata* L.) against *Bacillus subtilis* Test Bacteria.



BW-1LM BW-2LP BW-3LK

Figure 2: Secondary Antibacterial Activity Test Results of Endophytic Bacteria from Soursop Leaves (*Annona muricata* L.) against *Salmonella typhi* Test Bacteria.

3.3 Amplification of BW-1LM Isolate DNA by PCR

In prokaryotic organisms, there are three types of rRNAs with 5S, 16S, and 23S sedimentation coefficients. The 16S rRNA gene is a gene specific to the prokaryotic species (Clarridge, 2004). The 16S rRNA gene region is a subunit of the 30S prokaryotic ribosome that has 1542 nucleotide components. The 16S rRNA gene region is commonly used for bacterial identification and phylogenetic studies (Kumala, 2014).

Amplification of the 16S rRNA gene was performed on BW-1 LM endophytic bacterial isolate by PCR method and using a 27f primer (5'-AGA GTT TGA CTG GCT CAG-3 ') and 1492r primer (5'-TAC GGC TTA CCT TGT TAC GA- 3 ') as used by Rosita (2012). Primary 27f is a forward primer attached to the 5 'end of the previously decomposed target DNA strand, while the primary 1492r is the reverse primer that will be attached to the other end of the 5' single chain.

The amplification process successfully performed was marked by the presence of DNA fragments on the agarose gel, which can be seen in Figure 3. The magnitude of the DNA fragment is in the range of 1500 bp. This corresponds to a 16S rRNA gene that has a magnitude of about 1500 bp (Clarridge, 2004). The result of the amplicon that has been in electrophoresis for the sequencing process to know the order of nucleotide base. Sequencing is a technique used to sequence nucleotide bases in DNA fragments (Brown, 1995; Sambrook *et al*., 1989). The results of sequence data processing were analyzed using the nBLAST program online on the NCBI website (http://blast.ncbi.nlm.nih.gov/). The analysis was conducted with the aim of comparing sequenced data (query) from the results of research with DNA sequences from various corners of the world that were deposited and published on DNA banks or Gene Bank.

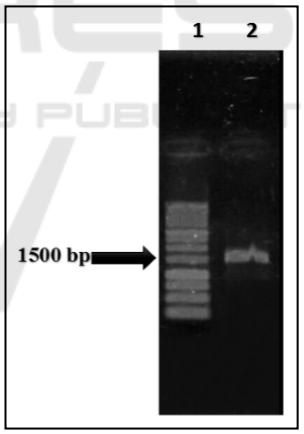


Figure 3: Result of Amplicon Electrophoresis DNA Isolate Bacteria Endophytes BW-1LM from Soursop Leaf. Lane 1: Ladder DNA 1kb Lane 2: product PCR.

Table 2: Measurement Results of Secondary Metabolite Constraint Zone Endophytic bacteria from Soursop Leaves against *Bacillus subtilis* and *Salmonella typhi*.

No.	Isolate Code	Bacteria	Antibacterial Activity		
			Day 5	Day 6	Day 7
1	BW-1 LM	*B.subtilis*	8,76mm	9,01mm	-
2	BW-1 LM	*S.typhi*	-	9,11mm	9,21mm

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus licheniformis strain DSM 13 16S ribosomal RNA gene, complete sequence	2678	2678	99%	0.0	99%	NR 118996.1
Bacillus licheniformis strain ATCC 14580 16S ribosomal RNA gene, complete sequence	2667	2667	99%	0.0	99%	NR 074923.1
Bacillus licheniformis strain BCRC 11702 16S ribosomal RNA gene, partial sequence	2610	2610	95%	0.0	99%	NR 116023.1
Bacillus licheniformis strain NBRC 12200 16S ribosomal RNA gene, partial sequence	2603	2603	95%	0.0	99%	NR 113588.1
Bacillus aerius strain 24K 16S ribosomal RNA gene, partial sequence	2588	2588	96%	0.0	99%	NR 042338.1
Bacillus sonorensis strain NBRC 101234 16S ribosomal RNA gene, partial sequence	2586	2586	95%	0.0	99%	NR 113993.1
Bacillus subtilis subsp. inaquosorum strain BGSC 3A28 16S ribosomal RNA gene, partial sequence	2545	2545	99%	0.0	97%	NR 104873.1
Bacillus subtilis strain 168 16S ribosomal RNA gene, complete sequence	2540	2540	99%	0.0	97%	NR 102783.1
[Brevibacterium] halotolerans strain DSM 8802 16S ribosomal RNA gene, complete sequence	2534	2534	99%	0.0	97%	NR 115063.1
Bacillus subtilis strain IAM 12118 16S ribosomal RNA gene, partial sequence	2534	2534	99%	0.0	97%	NR 112116.1
Bacillus subtilis strain DSM 10 16S ribosomal RNA gene, partial sequence	2534	2534	99%	0.0	97%	NR 027552.1
Bacillus mojavensis strain IFO15718 16S ribosomal RNA gene, partial sequence	2534	2534	99%	0.0	97%	NR 024693.1
Bacillus vallismortis strain DSM 11031 16S ribosomal RNA gene, partial sequence	2523	2523	99%	0.0	97%	NR 024696.1
Bacillus atrophaeus strain JCM 9070 16S ribosomal RNA gene, partial sequence	2516	2516	99%	0.0	97%	NR 024689.1
Bacillus amyloliquefaciens subsp. plantarum strain FZB42 16S ribosomal RNA gene, complete sequence	2512	2512	99%	0.0	97%	NR 075005.1
Bacillus sonorensis strain NRRL B-23154 16S ribosomal RNA gene, partial sequence	2507	2507	93%	0.0	99%	NR 025130.1
Bacillus nematocida strain B-16 16S ribosomal RNA gene, partial sequence	2490	2490	99%	0.0	97%	NR 115325.1
Bacillus subtilis strain JCM 1465 16S ribosomal RNA gene, partial sequence	2475	2475	95%	0.0	98%	NR 113265.1
Bacillus subtilis strain NBRC 13719 16S ribosomal RNA gene, partial sequence	2475	2475	95%	0.0	98%	NR 112629.1
Bacillus subtilis subsp. spizizenii strain NBRC 101239 16S ribosomal RNA gene, partial sequence	2475	2475	95%	0.0	98%	NR 112686.1
Bacillus tequilensis strain 10b 16S ribosomal RNA gene, partial sequence	2473	2473	96%	0.0	97%	NR 104919.1

Figure 4: Description of Nucleotide Results BLAST Gen 16S rRNA Bacterial Isolate Endophytes from Soursop Leaf.

Criteria that meet the taxonomic requirements are said to be the same species if the database sequence has an identity percentage between 95% - 99% and can be defined at the species level if the identity percentage is ≥99% (Clarridge, 2004). Figure 4 is the result of sequence alignment analysis with the nBLAST program. Result of the data shows that the sample of BW-1LM isolate has zero E-value and the same similarity percentage is 99% in bacterial species of *Bacillus licheniformis* strain DSM 13. The zero value expresses the higher level of trust that the query sequence has high homology level with database sequence. So the endophytic bacterial isolates in this study can be named *Bacillus licheniformis* strain BW-1LW.

4 CONCLUSION

It can be concluded that this research was successfully obtained three endophytic bacterial isolates from soursop leaf. However, only one of them has the antibacterial activity against *Bacillus subtilis* and *Salmonella typhi*. The result of molecular identification with PCR technique showed that BW-1LW isolate had 99% similarity to *Bacillus licheniformis* DSM 13 strain.

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