

Antibacterial activity of endophytic fungi in *Pometia pinnata* against *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus*

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Abstract. Setyaningsih R, Susilowati A, Prasetyani D. 2020. Antibacterial activity of endophytic fungi in *Pometia pinnata* against *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus*. *Biodiversitas* 21: 5408-5413. Endophytic fungi associated with medicinal plants are able to produce bioactive compounds which potentially contain medicinal ingredients. Matoa (*Pometia pinnata* J. R. Forst & G. Forst) is one of Indonesian medicinal plants. This research aimed to test antibacterial activities of ethyl acetate extract of *P. pinnata*'s endophytic fungi to *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA) and also identify isolates having high antibacterial activity. Antibacterial activity test was conducted by using paper disc diffusion. The fungi were identified in morphological and molecular manner with amplification and sequencing of ITS region. Bacterial cells exposed to the extract were observed by using scanning electron microscope (SEM). Isolate AM72 was found out to have the highest antibacterial activity compared to other 12 isolates tested, with inhibition zone diameter of 31.57 mm to *S. aureus* and 22.87 mm to MRSA. Isolate AM72 was identified as *Lasiodiplodia theobromae*. Isolate BMB identified as *Aspergillus oryzae* produced inhibition zone with 21.96 mm diameter to *S. aureus* and 21.50 mm diameter to MRSA. Antibacterial compounds produced by isolate AM72 were found out to be able to damage bacterial cell walls.

Keywords: Antibacterial, endophytic fungi, *Pometia pinnata*, *Staphylococcus aureus*, MRSA

INTRODUCTION

Staphylococcus aureus is an opportunistic pathogen in the human body, especially in the skin surface and nasal membrane. This bacterium can cause a variety of nosocomial infections, ranging from skin irritation, food poisoning, to sepsis, osteomyelitis, and endocarditis. One of the phenomena of bacterial resistance to antibiotics is the existence of methicillin-resistant *Staphylococcus aureus* (MRSA) bacteria (Chukwunonso et al. 2018). MRSA is *S. aureus* strain resistant to β -lactam antibiotics, including penicillin, methicillin, dicloxacillin, nafcillin, oxacillin, and cephalosporins (El-Gayar et al. 2014). Resistance usually occurs because bacteria acquire a non-native gene coding for penicillin-binding protein (PBP2a), with a much lower affinity for β -lactam (Peacock and Paterson 2015). This phenomenon of resistance pushes for the efforts of searching for new antibacterial compounds for medical treatment.

Endophytic fungi are potential sources of new antibiotics because of the variety of bioactive compounds it produced. Endophytic refers to the microorganisms colonizing the intercellular and intracellular areas of healthy plant tissues at a certain time and whose presence is unobtrusive and asymptomatic (Alvin et al. 2013). Based on the existing research, endophytic fungi can produce bioactive compounds that can function as antibiotics, such as terpenoids, steroids, alkaloids, phenols, flavonoids, polyketides, and aliphatic compounds (Mousa and Raizada 2013). The relationship between endophytic fungi and host plants can affect the formulation of bioactive compounds

produced (Jia et al. 2016).

One of the plants widely used for its medicinal properties is matoa (*Pometia pinnata* J.R. Forst & G. Forst). *P. pinnata* is used as medicine by traditional communities in Manokwari, Western Papua Province, Indonesia. The community uses its stems to treat burns and incision wounds (Lense 2012). Its bark extract has inhibition power to gram-positive bacteria like *S. aureus* because of its tannin, flavonoid, terpenoid, and saponin contents (Ngajow et al. 2013). Because of these medicinal properties contained in *P. pinnata* plant, further research is needed in relation to endophytic fungi of *P. pinnata* having antibacterial activity. This research aimed to test antibacterial activities of ethyl acetate extract of *P. pinnata*'s endophytic fungi to *S. aureus* and Methicillin-resistant *Staphylococcus aureus* (MRSA), and also identify isolates having high antibacterial activity. Utilization of endophytic fungi as source of bioactive compounds brings more benefit because of its suitability for mass-scale cultivation so that a large amount of bioactive compounds can be harvested in a relatively short amount of time without causing threat to the plant's conservation.

MATERIALS AND METHODS

Endophytic fungi isolates

Thirteen isolates of endophytic fungi were selected for testing. The selected fungi had quick growth as their main feature. Colonies having quick growth can grow filling

agar surface in petri dish of 9 cm diameter in a week. The thirteen isolates came from roots, stems, and leaves of *P. pinnata* (Table 1).

Table 1. Endophytic fungi isolated from roots, stems, and leaves of *Pometia pinnata* having fast growth rate

Source of isolates	Name of isolates	Number of isolates
Roots	AM2, AM4, AM6, AM71, AM72	5
Stems	BM5, BM6, BME, BM1B, BMB	5
Leaves	DM1, DM2, DM6	3
Total number of isolates		13

Endophytic fungi isolates and bioactive compounds extraction

Thirteen isolates of *P. pinnata* endophytic fungi collection of Laboratory of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret, Surakarta, Indonesia were rejuvenated in potato dextrose agar (PDA) media. The fungi were isolated from a healthy plant of *P. pinnata* from Karangpandan, Karanganyar District, Central Java Province, Indonesia using isolation method described by Marcellano et al. (2017). Extraction of bioactive compounds was conducted based on Sharma et al. (2016) with modifications. Endophytic fungi were cultivated in 200 ml of potato dextrose broth (PDB) and incubated in incubator shaker Infors HT Ecotron with 90 rpm speed on 28°C for 14 days. After 14 days, liquid cultures were filtered by using filter paper to separate mycelium and the media. The extraction was then conducted by using ethyl acetate, by adding 100 ml of ethyl acetate to mycelium and 100 ml of ethyl acetate to the filtered media, and then macerated for 24 hours. Mycelium was then separated from ethyl acetate by filtering using filter paper. The filtrates obtained were separated by using separating funnel to obtain bioactive compounds in the ethyl acetate.

Antibacterial test by disc diffusion method

An amount of 100 µL of clinical isolated *S. aureus* cultured in PDB with 0.5 turbidities in McFarland standard was cultured in Mueller-Hinton agar (MHA) by using spread-plate method, and then was left for a few moments. An amount of 30 µL of the pure extract was dripped to paper disc on bacteria-inoculated MHA surface. Afterward, the culture was incubated for 24-48 hours at 37°C. Antibacterial activities were shown by the existence of zone of inhibition around the disc. 30 µL of 2 mg/mL chloramphenicol was used as positive control and ethyl acetate was used as negative control (Atiphasaworn et al. 2017). Isolate extracts showing inhibition activity to *S. aureus* were then tested for their inhibition to clinical isolated MRSA. Extracts of isolates having largest zone of inhibition diameter to *S. aureus* and MRSA were used in next test stages.

Endophytic fungi morphological identification

Fungi identification was conducted by observing their macroscopic and microscopic characteristics. Macroscopic

observation was conducted by directly observing fungi colonies growing on PDA media surface. Characteristics observed include the colony's upper and lower surface color, the colony's surface texture, the colony's edge, and the colony's ability to change media color. Microscopic observation used slide-culture method. Characteristics observed include septa's existence on the hyphae, the hyphae's color, the shape of conidia or sporangium, and special characteristics determining the fungi type. Macroscopic and microscopic identification of fungi was conducted based on Watanabe (2002).

Endophytic fungi molecular identification

Fungi isolates were cultured on PDB media and incubated for 3 days in shaker incubator with 28°C temperature and 90 rpm rotation. An amount of 50-100 mg wet weight of mycelium growing in the media was used for genome DNA extraction, by using Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, Irvine, USA), with stages of extraction were conducted in accordance with factory procedure. Extracted DNA's quality was then tested by using biophotometer (Jin et al. 2004), and amplified by using polymerase chain reaction (PCR) method, using primer ITS1 (5'TCC GTA GGT GAA CCT GCG G 3') and ITS4: (5' TCC TCC GCT TGA TAT GC 3') to amplify ITS region. PCR reaction was conducted for 50 µL in total, consisting of 5 µL of DNA template, 2.5 µL of respective forward and reverse primer (20µM), 25 µL of MyTaq HS Red Mix, and 15 µL of nuclease-free water. PCR (Eppendorf Mastercycler Personal) condition for ITS was set to: predenaturation on 95°C for 1 minute, denaturation on 95°C for 15 seconds, annealing on 59°C for 15 seconds, DNA elongation on 72°C for 10 seconds, 35 times of cycle repeat, final extension on 72°C for 7 minutes and final hold on 4°C. PCR results were migrated on 1% agarose gel and visualized on GelDocumentation. Amplified DNA was then sequenced. Obtained base sequence of amplified DNA was subsequently compared to the DNA base sequence existing in NCBI GenBank (<http://www.ncbi.nlm.nih.gov/blast>) to identify fungal species.

Observation of MRSA bacterial cells exposed to extract through Scanning Electron Microscope (SEM)

Ethyl acetate extract with highest antibacterial activity was then exposed to MRSA and observed by using SEM (Supaphon et al. 2013). SEM machine used was Hitachi SU3500. One hundred µL of extract with 6,25% (v/v) concentration were added to 900 µL of bacterial suspension and mixed using vortex. Then the mixture was incubated at 37 °C for 18 hours. Pellets were then fixated with 2.5% glutaraldehyde (C₃H₈O₂) for 2 hours, and then washed 2 times with phosphate-buffered saline (PBS). Pellets were consequently post-fixated by using 1% osmium tetroxide for 1 hour and washed again by using PBS. After that, pellets were dehydrated by using alcohol in stages, with 50%, 70%, 80%, 90%, and 100% concentration. Pellets were then freeze-dried and analyzed by using SEM.

RESULTS AND DISCUSSION

Endophytic fungi antibacterial activity to *Staphylococcus aureus* and MRSA

Ethyl acetate extract of 8 cultures of endophytic fungi isolates was found out to have antibacterial activity, while 5 isolates were found out not to produce antibacterial

compounds to *S. aureus*. Antibacterial activity is marked by the formation of clear zones around the paper disc showing the existence of bacteria growth inhibition (Figure 1). Isolates AM72, DM2, BMB, DM1, AM71, AM4, DM 6, and AM2 produced inhibition zone with diameters approximately between 8 mm-31.57 mm (Table 2).

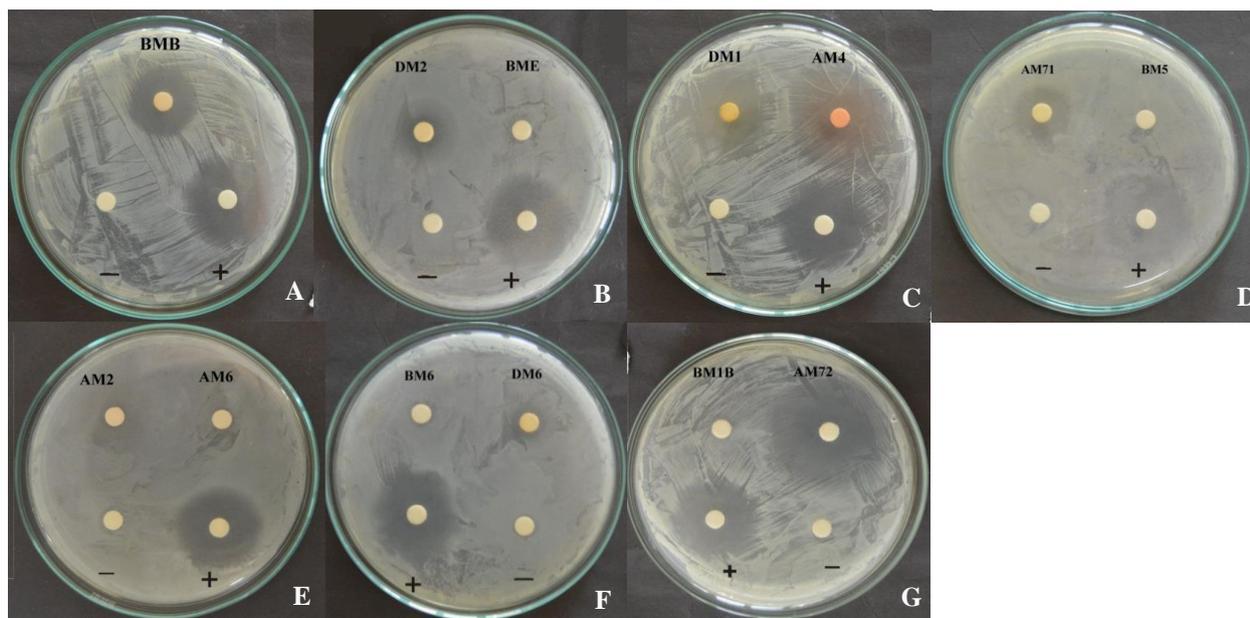


Figure 1. Inhibition zone formed by ethyl acetate extracts of endophytic fungi isolated from roots, stems, and leaves of *Pometia pinnata* to *Staphylococcus aureus*. Zones formed by extracts (30 μ L) of isolate: A. BMB, control +, control -, B. DM2, BME, control +, control -, C. DM1, AM4, control +, control -, D. AM71, BM5, control +, control -, E. AM2, AM6, control +, control -, F. BM6, DM6, control +, control -, G. BM1B, AM72, control +, control -

Table 2. Antibacterial compound inhibition zone diameter in ethyl acetate extracts of *Pometia pinnata*'s endophytic fungi to *S. aureus*

Name of isolates	Diameter of inhibition zone (mm) of 30 μ L extract
AM72	31.57
DM2	22.83
BMB	21.96
DM1	20.95
AM71	18.40
AM4	17.66
DM6	13.50
AM2	8.00
BM6	0
AM6	0
BM1B	0
BM5	0
BME	0
Positive control (Chloramphenicol 20mg/mL)	27.47
Negative control (ethyl acetate)	0

Isolate AM72 produced antibacterial compounds forming largest inhibition zone, which amounted to 31.57 mm. Chloramphenicol which was used as a positive control was a broad-spectrum antibiotic (Dinos et al. (2016). Negative control in form of ethyl acetate did not produce inhibition zone, meaning that the extract's inhibition to bacteria was not influenced by solvent during extraction. Extracts showing inhibition power to *S. aureus* were then tested to MRSA. Ethyl acetate extracts of *P. pinnata*'s endophytic fungi having antimicrobial activity to MRSA were shown by the existence of inhibition zone with approximate diameters between 11.01-22.87 mm. Compounds produced by isolate AM72 had inhibition power with the largest diameter, however, the inhibition zone's size was still smaller than the inhibition zone formed by positive control (vancomycin 30 μ g) (Figure 2 and Table 3). Vancomycin is an antibiotic that is often used to treat MRSA infections (Tang 2015).

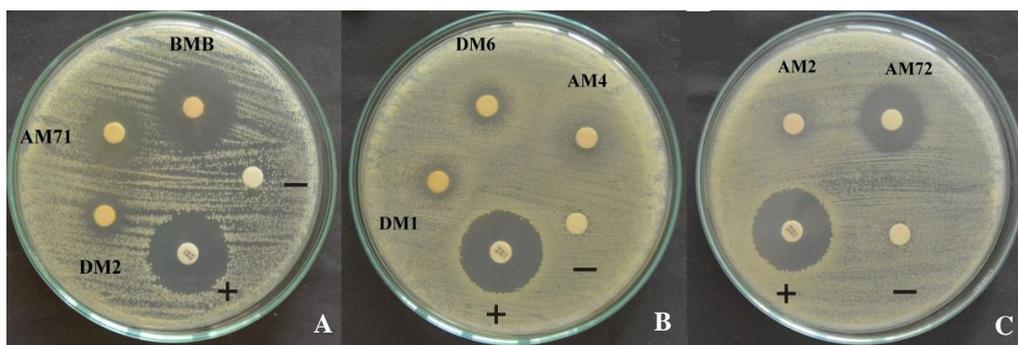


Figure 2. Inhibition zone formed by ethyl acetate extracts of endophytic fungi isolated from roots, stems, and leaves of *Pometia pinnata* to MRSA. Zones formed by extracts of isolate: A. DM2, AM71, BMB, control +, control -, B. DM1, DM6, AM4, control +, control -, C. AM2, AM72, control +, control -

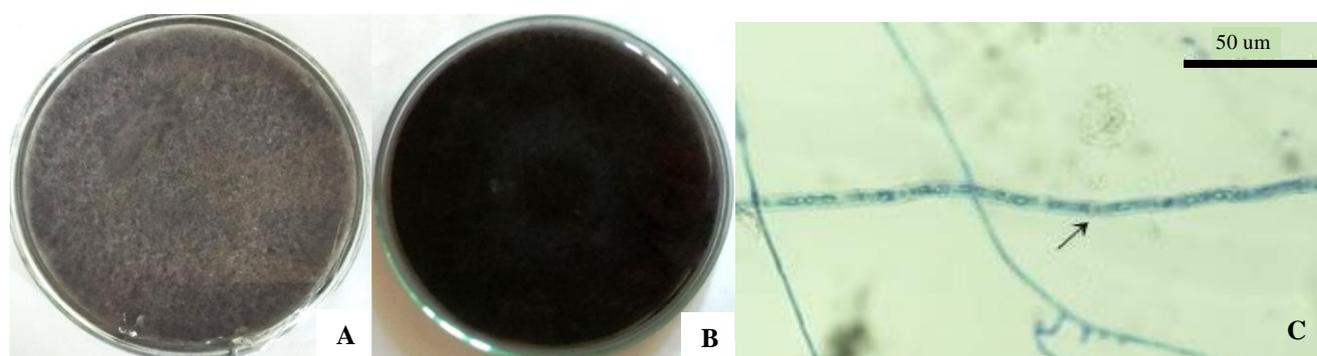


Figure 3. Isolate AM72 colony. A. Obverse of colony, B. Reverse of colony. C. Septate hyphae of Isolate AM72

Table 3. Diameter of antibacterial inhibition zone of ethyl acetate extracts of endophytic fungi roots, stems, and leaves of *Pometia pinnata* to MRSA.

Name of isolates	Diameter of inhibition zone (mm) of 30 µL extract
AM72	22.87
BMB	21.50
AM71	16.86
AM4	13.14
DM2	12.23
DM6	11.67
DM1	11.01
AM2	0
Positive control (vancomycin 30 µg)	24.69
Negative control (ethyl acetate)	0

Extracts of endophytic fungi culture from seven isolates showed antibacterial activity to MRSA, while extract from one isolate (AM2) having antibacterial activity to *S. aureus* but was found out not to inhibit MRSA. Inhibition zone formed by ethyl acetate extracts of endophytic fungi to MRSA test bacteria were found out to be lower than to *S. aureus*. This shows that *S. aureus* is more sensitive to antibacterial compounds found out in the extracts than MRSA. Inhibition power to *S. aureus* and MRSA were approximated to have 2.1-47% difference. Isolates DM2

and DM1 had relatively high inhibition power to *S. aureus*, but lower inhibition power to MRSA.

Identity of endophytic fungi producing anti-*Staphylococcus aureus* and anti-MRSA compounds

Two of thirteen endophytic fungi isolates that have highest antibacterial features to MRSA, isolates AM72 and BMB, were identified. Macroscopic characteristics of isolate AM72 on PDA media showed that the colony's upper surface was grayish in color, changing to black as it ages, cottony in nature with abundant air mycelium. Its lower surface was black (Figure 3.A-B). Isolate AM72 had microscopic characteristics of transparent hyphae (hyaline) and was septate, with no spores were present (Figure 3.C). Several researchers show that many endophytic fungi do not produce reproduction structures, or called having mycelia sterilia because of their failure in producing spores on culture media, so that a molecular approach is needed to identify endophytic fungi (Verma et al. 2019).

Isolate BMB is grayish-dark green in color with flat edges, while its middle part is wavy with cerebriform pattern. The colony has floury or powdery texture, with the reverse having yellow color. Through microscopic observation, isolate BMB has vegetative mycelium having septa and hyaline, with its conidiophores have no septa and hyaline, and its conidiospore having round shape (Figure 4.A-C). Based on the characteristics isolate BMB was grouped under *Aspergillus* genus.

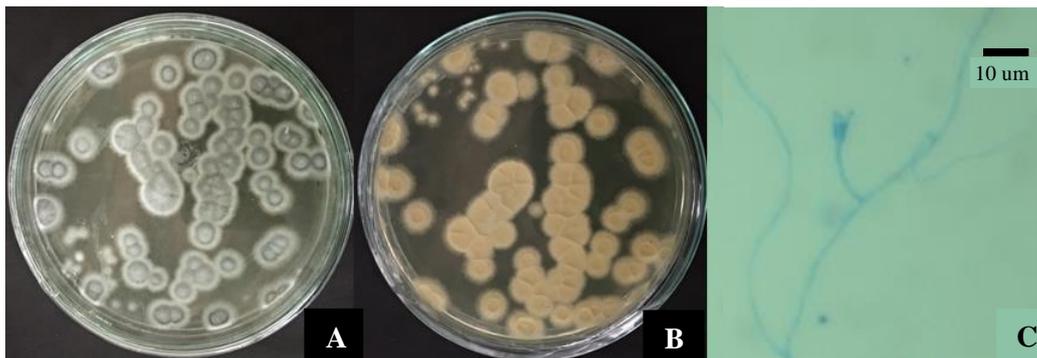


Figure 4. Isolate BMB morphology. A. Obverse of colony, B. Reverse of colony, C. Hyphae with conidiospore

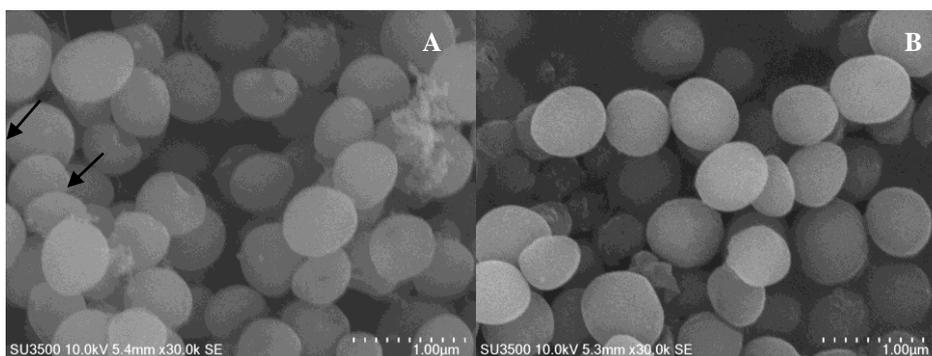


Figure 6. MRSA bacterial cells treated by *Lasiodiplodia theobromae* AM72 culture extract with 6.25% concentration (v/v) (A) and cell without treatment (B) shown by scanning electron microscope (SEM)

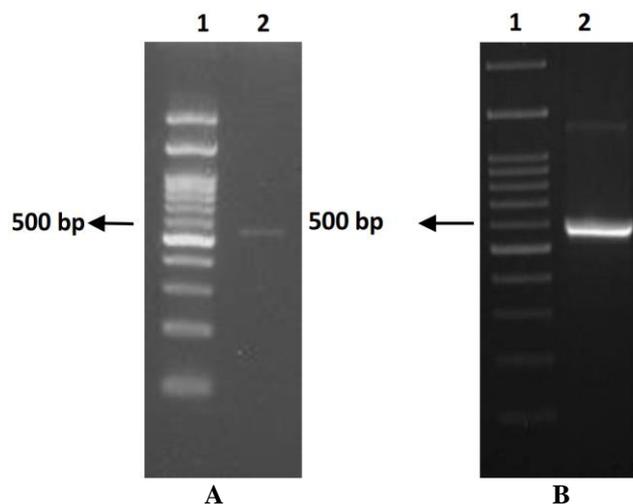


Figure 5. Amplicon of DNA fragment from ITS region of: A. Isolate AM72, B. Isolate BMB

Table 5. Identity of Isolates AM72 and BMB

Isolates	% Similarity	Query cover	Accession number
AM72	100% with <i>Lasiodiplodia theobromae</i> strain FV	99%	KX022498.1
BMB	99% with <i>Aspergillus oryzae</i> strain AD-B2	100%	Q285519.1

Molecular identification confirms results of morphological identification. Amplicon DNA fragments from ITS region of isolates AM72 and BMB were placed between the bands with a size of 500 and 600 bp (Figure 5). Based on BLAST-N analysis, nucleotide order of isolate AM72 had 100% similarity to *Lasiodiplodia theobromae* strain FV, while isolate BMB had 99% similarity to *Aspergillus oryzae* strain AD-B2 (Table 5). Based on morphological and molecular characteristics, isolate AM72 was identified as *L. theobromae*, while isolate BMB was identified as *A. oryzae*.

Lasiodiplodia theobromae is an anamorph form of *Botryosphaeria rhodina*. This fungus is an often-encountered endophyte and an opportunistic pathogen causing disease when its host is in unfavorable condition. *L. theobromae* can live in more than 500 species of tropical and subtropical plants (Darge 2017). *L. theobromae* isolated from the *Piper hispidum* Sw plant has antibacterial activity against some bacteria species (Orlandelli et al. 2012). Bioactive compounds produced by *L. theobromae* isolated from mangrove *Acanthus ilicifolius* in addition to having antibacterial activity also potentially inhibit human cancer cell lines (Chen et al. 2016). From several studies, it can be said that *L. theobromae* endophytic fungi are quite potential to be developed as a source of bioactive compounds because, in addition to their compound content, it is also easy to find life as endophytes in various plants

Members of the *Aspergillus* genus are widely known as endophytes. *Aspergillus* endophytes are the potential

source of new secondary metabolites with valuable biological activity (El-hawari et al. 2020). *A. oryzae* isolated from *Calotropis procera* plant showed antibacterial activity against *Escherichia coli*, *Klebsiella pneumoniae*, *S. aureus*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Shigella flexneri*, *Enterococcus faecalis* and *Proteus mirabilis* (Rani et al. 2017). The species of *Aspergillus* which has been known to have the most antibacterial activity is *A. fumigatus* (Chagas et al. 2017; Al-Fakih and Almaqtri 2019; El-Hawari et al. 2020

MRSA bacterial cell damage by antimicrobial compound in the extract

Staphylococcus cells have round shapes. Through SEM it can be seen that MRSA cells treated with ethyl acetate extract of *L. theobromae* AM72 culture undergone deformation of indentation of cells inwards (Figure 6). These indentations showed the existence of damage in peptidoglycan, which gives these cells their shape. According to Matijašević et al. (2016), biosynthesis and the formation of peptidoglycan crosslinking are regulated by the cytoplasmic membrane, so that changes in the shape of the cell surface as seen in Figure 6 may be caused by damage of the cytoplasmic membrane resulting in peptidoglycan damage.

The research gave results that largest antibacterial activity was found out to be owned by isolate AM72, identified as *L. theobromae*. Bacterial cells treated with the extract showed signs of damage, demonstrated by the formation of inward indentation in the cells. Endophytic fungal isolates from *P. pinnata*, especially *L. theobromae* AM72 have potential to be further developed as a source of antibacterial medicine, especially for strains resistant to antibiotics commonly used. Besides *L. theobromae* AM72, isolate BMB (*A. oryzae*) needs further research on its antibacterial activity.

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