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Characterization, identification, and analysis of bioactive compound of endophytic bacteria from *Hoya multiflora* Blume

DEWINTA NUR ALVIONITA[1,♥], SRI RAHAYU[2], NISA RACHMANIA MUBARIK[1,♥♥]

[1]Department of Biology, Faculty of Mathematics and Natural Sciences, Institut Pertanian Bogor. Jl. Agatis, IPB Dramaga Campus, Bogor 16680, West Java, Indonesia. Tel./fax.: +62-251-8622833, ♥email: nonyalvionita@gmail.com, ♥♥ nrachmania@apps.ipb.ac.id

[2]Research Center for Plant Conservation Botanic Gardens, Indonesian Institute of Sciences. Jl. Ir. Juanda 13, Bogor 16122, West Java, Indonesia

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Abstract. *Alvionita DN, Rahayu S, Mubarik NR. 2020. Characterization, identification, and analysis bioactive compound of endophytic bacteria from* Hoya multiflora *Blume. Biodiversitas 21: 195-202. Hoya multiflora* Blume has been used for various purposes. Extract hoya has been used antibacterial, stomachache, skin diseases and natural insecticidal. Extract hoya has been reported to have bioactivity but extraction from the plant has been inefficient because it requires large biomass. Therefore, one of the appropriate ways to extract its bioactive compounds is by using endophytic bacteria. The purposes of this research were to isolate, characterize the endophytic bacteria from *H.* multiflora, identify its bioactive compound and to test its antimicrobial activity against two pathogenic bacteria. A total of 18 isolates from *H. multiflora* Blume were successfully obtained. Based on antimicrobial test, isolate HMA 2 was able to inhibit *Escherichia coli*, isolate HMD 6 inhibited *Staphylococcus aureus* and two isolates inhibited both of pathogenic bacteria (i.e isolate HMB 1 and HMD 4). In addition, minimum inhibitory concentration (MIC) of ethyl acetate extract from HMB 1 was 0.625 mg/mL, while from HMD 4 was 0.125 mg/mL, both against *E. coli* and *S. aureus*. Based on 16S rRNA gene sequencing analysis, HMB 1 was the most similar (99.91%) with *Bacillus siamensis* KCTC-13613 and HMD 4 was the most similar (100%) with *Bacillus aryabhattai* B82W22. Using GC-MS and compared with database WILLEY9THN08, isolate HMB 1 identified compounds such as 3-benzyl-1,4-diaza-2,5-dioxobicyclo and 13 Docosenamide, isolate HMD 4 contained compounds such as linolenic acid, 1,2-benzene dicarboxylic acid, oleic acid amide, 1-nonadecene, and stigmasterol. More than, there are 3 compound identified from isolates HMB 1 and HMD 4: phenol, 2,4-bis (1,1-dimethyl); neophytadiene dan pyrollo [1,2-a]pyrazine-1,4-dione,hexahydro-3- (phenylmethyl), respectively.

Keywords: 16S rRNA, antimicrobial, bioactive compound, endophytic bacteria, MIC

INTRODUCTION

Hoya multiflora Blume is one of the 499 genera in family Apocynaceae (Endress and Stevens 2001). It is generally epiphytic, thick-leafed and has star-shaped flower. It is a medicinal herb and peoples used it for stomachache, cough, toothache, venereal diseases, skin decease, and antitoxin (Rahayu 2011). *H. multiflora* has the widest geographical occurrences, covering 80% of the distribution of Genera *Hoya* (Rintz 1980). Several studies proved that Hoya extract can be used as antibacterial and antinociceptive agent (Reza et al. 2007), *Hoya carnosa* leaf extract as antibacterial for chronic media otitis patients (Rahayu et al. 2017).

It is generally found that bioactivities of plants are inseparable from their compounds. Extract hoya has been reported to have bioactivity but extraction from the plant has been inefficient because it requires large biomass *Hoya diversifolia* contains terpenoid (Warnaar 1984), tryptone and sterols (Nelson et al. 2015). Latest study revealed that the stem of *H. multiflora* contains, *β*-lupeol, α-amyrin, *β*-amyrin, lupeol acetate, α-amyrin acetate, and β-amyrin acetate, and the leaf contains α-amyrin, bauerenol, squalene, lutein, *β*-sitosterol, and stigmasterol (Ebajo et al. 2015). Extract hoya has been reported to have bioactive compound but extraction from the plant has been inefficient because it requires large biomass. Therefore, one of the appropriate ways to extract its bioactive compounds is by using endophytic bacteria. The presence of active compounds in plants is presumably closely related to the presence of endophytes including bacteria. Such bacteria in plant tissue can be neutral for or benefit the host plant (Bhore and Sathisha 2010). Several studies have demonstrated the role of endophytic bacteria in inhibiting other microorganisms. Endophytic bacteria on the leaves of *Coleus scutellariodes* [L.] Benth are reported capable to inhibiting the growth of *Escherichia coli* and *Staphylococcus aureus* (Kusumawati 2014), *Bacillus subtilis* isolated from mulberry showing antimicrobial activity (QiongYing et al. 2012), *Bacillus pumilu*s MAIIM4 can inhibit the growth of *Rhizoctonia solani* and *Sclerotium rolfsii (*Melo et al. 2009) and Jose and Christy (2013) reported that *Serratia*, *Bacillus*, *Pseudomonas*, *Micrococcus*, and *Enterobacter* from *Rhizophora mucronata* are able to produce antimicrobial compounds that are active against bacterial and fungal pathogens.

The endophytic bacteria and plant hosts share mutualistic symbiosis. In this case, endophytic bacteria obtain nutrients from plant's metabolic product whilst protecting the plants against pathogens. As for the plant, it gains nutrient derivates and active compounds it requires for life. However, there had been no study on the influence of endophytic bacteria from hoya as antimicrobial and bio-active compounds of endophytic bacteria from *H. multiflora.*

Therefore, a study was necessary to understand endophytic bacteria from hoya by isolating, characterizing, and identifying the bacteria based on 16S rRNA, antimicrobial activity and identification of bioactive compounds using Gass chromatography-mass spectrophotometry (GC-MS).

MATERIALS AND METHODS

Sample collection and endophytic bacteria isolation

H. multiflora was obtained from Hoya collection of Bogor Botanical Garden. Only healthy plants were taken sample preparation for isolation of endophytic bacteria from various plant parts (root, stem, and leaf). Plant tissues were surface-sterilized according to Desriani et al. (2013) with some modifications. Fresh samples were cleaned under running water, cut off 1-3 cm each, and grouped by the plant's part. Samples were soaked in 70% alcohol for 1 minute, then soaked in 1% sodium hypochlorite (NaOCl) for 3 minutes, then in 70% alcohol for 1 minute, and rinsed using sterile distilled water thrice. Sterile samples were grounded using mortar and pestle 0.1 mL of each were taken. The samples were then inoculated on Nutrient Agar (NA) prior to incubation for 24-48 hours. Surface sterilization is successful when control agar grows no bacteria. Endophytic bacteria that grew on the media were purified one by one, characterized and cultivated on slant agar.

Antimicrobial activity assay

One loop of each tested bacterial inoculant (*E. coli* and *S. aureus*) from slant agar stock was taken and grown in 20 mL Nutrient Broth (NB) prior to incubation in orbital shaking incubator at 37 °C for 18 hours. 1% of the bacteria was then put into 250 mL (Nutrient Agar) NA and poured into petri dish to solidify. Antimicrobial activity of endophytic bacteria was tested following paper disc method using bacteria supernatant. One loop of endophytic bacteria was inoculated in 20 mL NB for 24 hours and put in shaker at room temperature ±27 °C prior to centrifugation for 15 minutes at 5000 rpm using centrifuge (HERME Labortechnik GmbHZ326K). Paper disc was put on the surface of NA media that was inoculated with 1% tested bacteria (*E. coli* and *S. aureus*). 13 µl supernatant from centrifugation was dropped on the paper disc prior to incubation for 24 hours at room temperature. Endophytic bacteria have potential as antimicrobial agent forms around the disc.

Determining minimum inhibitory concentration (MIC)

MIC of bacterial crude extract was determined following agar diffusion method according to Mazollla et al. (2009) with some modifications. Bacterial extract was made into initial stock solution (10000 ppm) by weighing 0.01 crude gram extract bacteria before dissolving in 1 mL Dimethyl Sulfoxide (DMSO). Stock solution was then made into 1 mg/mL and diluted into 6 serial concentrations (1 mg/mL 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL, 0.625 mg/mL dan 0, 312 mg/mL). Paper disc (Whatman) was then put on NA media that was inoculated with 1% tested bacteria (*E. coli* and *S. aureus*). 13 µl crude extract of 6 serial concentrations was then dropped onto the paper disc. Chloramphenicol (30µg) was used as positive control while DMSO was used negative control. Incubation was carried out at room temperature for 24 hours. MIC was determined by observing extract's lowest concentration that can inhibit bacteria.

Extracting and identifying microbial compounds from endophytic bacteria

Extraction and identification potential endophytic bacterial compounds were carried out according to Prastya et al. (2018) with some modifications. One loop endophytic bacteria inoculation was done into 30 mL NB and incubating in rotary shaker for 24 hours at room temperature. 1% culture was then put into 1000 mL Erlenmeyer containing 500 mL NB in rotary shaker for 3x24 hours at 100 rpm at room temperature. Bacterial supernatant was then extracted by adding 500 mL ethyl acetate (ratio 1: 1) to separatory funnel prior to shaking for 30 minutes, producing two layers, i.e. supernatant and ethyl acetate. The extract of bacterial compounds was then evaporated using rotary evaporator to obtain bacterial crude extract. Antimicrobial compound was identified using GC-MS by dissolving 10 mg bacterial crude extract in 1 mL ethyl acetate before injecting 0.1 µl of which into 0.25x25 mm capillary column (GC-17A, Japan). Stationary phase used 5% phenyl methyl siloxane at 1 ml/minute flow rate and helium was used as the carrying gas at 20 ml/minute flow rate.

Molecular characterization

Two isolates with highest antimicrobial activity were inoculated in tube containing 30 mL NB prior to growth for 24 hours at room temperature in shaker. The culture was then transferred into 1.5 ml tube and centrifuged at 13000 rpm for 1 minute with 3 replications. The supernatant was discarded and DNA in the pellet was extracted using Geneaid kit. DNA purity and concentration were then measured using Nanodrop 2000 spectrophotometer (Thermo Scientific, USA). 1 pmol DNA sample was added with 5 µl GoTaq Green Master Mix 2x, and 1 pmol primer 63F, and 1 pmol primer 1387R. Identification was carried out by amplifying gene encoding 16S rRNA using primer 63F (5'-CAG GCC TAA CAC ATG CAA GTC-3') and 1387R (5'-GGG CGG WGT GTA CAA GGC-3') on PCR machine (Marchesi et al. 1998). The PCR

reaction was carried out under the following conditions: pre-denaturation at 94 °C for 4 minutes, followed with 35 cycles comprising denaturation at 94 °C for 30 seconds, annealing at 55 °C for 45 seconds, and elongation at 72 °C for 1 minute 30 seconds. PCR process was ended by post-elongation at 72 °C for 10 minutes and cooling at 4 °C for 5 minutes. PCR product was electrophoresed in 1% agarose gel with buffer TAE 1x at 90 V for 60 minutes. DNA bands were stained using 5 µg/ml Ethidium Bromide (EtBr) and visualized using UV rays. PCR products were sequenced by PT Genetika Science (Jakarta, Indonesia).

DNA sequences were analyzed and its homology was determined by GenBank using Basic Local Alignment Search Tool (BLAST) program at http: //www.ncbi.nmL.nih.gov. MEGA ver. X application was employed to develop the phylogenetic tree, by attaching a reference sequence from BLAST result. Phylogenetic tree was developed following Maximum likelihood method and Tamura-2 parameter with bootstrap (BS) 1000x.

RESULTS AND DISCUSSION

Isolation and morphological characterization of endophytic bacteria

A total of 18 endophytic bacterial isolates were successfully obtained from the roots, stems, and leaves of *H. multiflora*. The isolates were then purified, resulting in 3 isolates from root, 8 isolates from stem, and 7 isolates from leaves. The isolates were then characterized manually (Table 1).

Antimicrobial activity of endophytic bacterial isolates against pathogenic bacteria

Endophytic bacterial isolates were tested for their antimicrobial activity by following disc diffusion method and observed for formation of clear zone formed around discs indicates antimicrobial activity. All 18 endophytic bacterial isolates were tested against 2 pathogenic bacteria, i.e. *E. coli* and *S. aureus* (Table 2). Based on antimicrobial assay, a total of 4 isolates were capable of inhibiting pathogenic bacteria, i.e. isolate HMA 2 against *E. coli*, isolate HMD 6 against *S. aureus*, and isolate HMB 1 and HMD 4 against both (*E. coli* and *S. aureus*) (Figure 1).

Determination of minimum inhibitory concentration (MIC)

Crude extracts obtained from two isolates with highest antimicrobial activity were determine MIC. Endophytic bacteria's metabolite crude extracts indicated different antimicrobial activities. Based on MIC test, 62.5 ppm was the lowest concentration of isolate HMB 1 crude extract to inhibit *E.coli* (diameter 3.6 mm), and 125 ppm was the lowest concentration of HMD 4 to inhibit *E.coli* (diameter 0.53 mm). In addition, 62.5 ppm HMB 1 was able to inhibit *S. aureus* at diameter 2.77 mm, and 125 ppm HMD 4 was able to inhibit the bacteria at diameter 0.4 mm (Figure 2).

16S rRNA gene identification

Two potential endophytic bacterial isolates from *H. multiflora* identified on molecular basis using 16S rRNA gene (Table 5). Based on BLAST NCBI, isolate HMB 1 showed the highest similarity (99.91%) with *B. siamensis* strain KCTC 13613 and isolate HMD 4 showed the highest similarity (100%) with *B. aryabhattai*. Both isolates were *Bacillus* for the phylogenetic relationship between isolate HMD 4, HMB 1, and other bacteria from the same genus (Figure 2).

Compound identification using GC-MS

The results of GC-MS analysis of ethyl acetate extract of isolates HMB 1 dan HMD 4 obtained chromatograms of compounds in the form of peaks and retention time. Components of bioactive compounds with a similarity quality of 90 - 99% with a percentage of > 1% and potential as antimicrobials from isolates HMB (Table 3) and HMD 4 (Table 4).

Table 1. Colony morphological characteristics of *H. multiflora* Blume endophytic bacterial isolates

Isolate code	Colony morphological characteristic					
	Form	Size	Surface	Texture	Color	Elevation
HMA 1	Circular	Small	Smooth	Mucoid	White	Flat
HMA 2	Irregular	Large	Smooth	Mucoid	White	Flat
HMA 3	Irregular	Medium	Shiny	Moist	White	Convex
HMB 1	Irregular	Large	Veiny	Dry	White	Flat
HMB 2	Circular	Medium	Smooth	Mucoid	White	Flat
HMB 3	Circular	Medium	Smooth	Mucoid	Yellow	Flat
HMB 4	Circular	Small	Smooth	Moist	White	Flat
HMB 5	Circular	Small	Smooth	Moist	Transparent	Flat
HMB 6	Circular	Medium	Smooth	Mucoid	White	Flat
HMB 7	Irregular	Medium	Veiny	Dry	White	Convex
HMB 8	Circular	Small	Smooth	Mucoid	Yellow	Flat
HMD 1	Circular	Small	Smooth	Mucoid	Transparent	Flat
HMD 2	Circular	Small	Smooth	Mucoid	White	Flat
HMD 3	Circular	Medium	Shiny	Moist	White	Convex
HMD 4	Irregular	Large	Shiny	Moist	White	Convex
HMD 5	Circular	Medium	Smooth	Moist	Transparent	Flat
HMD 6	Circular	Medium	Smooth	Mucoid	White	Flat
HMD 7	Circular	Small	Smooth	Moist	Yellow	Flat

Note: The tree letters of isolate code indicate the origin of endophytic bacteria from roots (A), Stems (B), leaves (D)

Table 2. Endophytic bacteria's antimicrobial activity against pathogenic bacteria

Isolate code	Inhibition zone (mm)		Isolate code	Inhibition zone (mm)	
	E.coli	*S.aureus*		*E. coli*	*S. aureus*
HMA 1	-	-	HMB 7	-	-
HMA 2	2.8± 0.1	-	HMB 8	-	-
HMA 3	-	-	HMD 1	-	-
HMB 1	3.1±0.1	4.1± 0.2	HMD 2	-	-
HMB 2	-	-	HMD 3	-	-
HMB 3	-	-	HMD 4	2.7±0.2	3.1±0.1
HMB 4	-	-	HMD 5	-	-
HMB 5	-	-	HMD 6	-	2.6 ± 0.3
HMB 6	-	-	HMD 7	-	-

Note: The tree letters of isolate code indicate the origin of endophytic bacteria from roots (A), Stems (B), leaves (D)

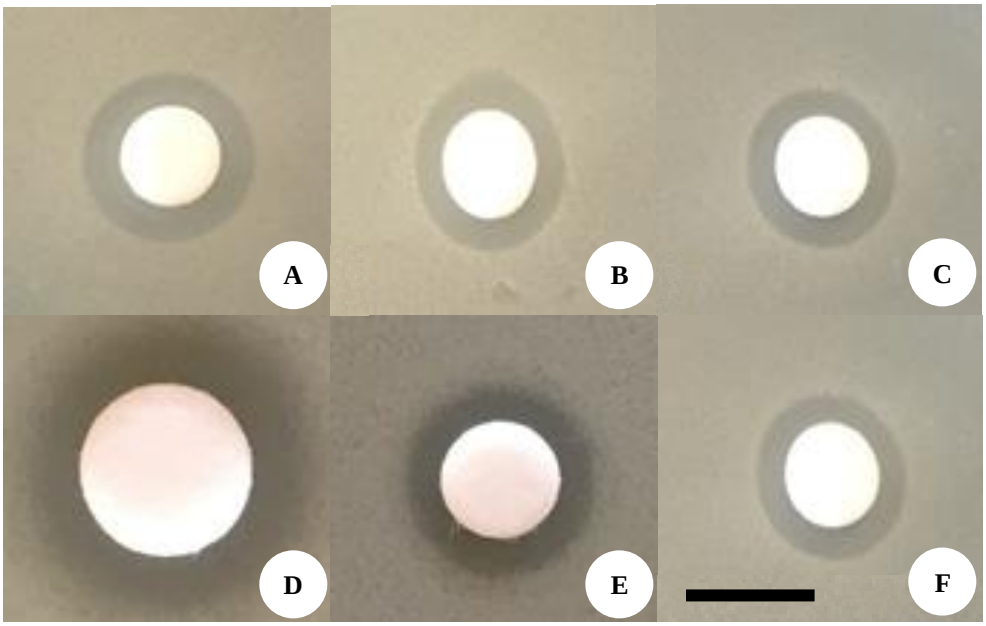

Figure 1. The results of antimicrobial activities of bacteria isolate isolated from endophytic bacteria of *H. multiflora* Blume. A: The inhibition of HMA 2 isolate on *E. coli*, B: The inhibition of HMB 1 isolate on *E. coli*, C: The inhibition of HMD 4 isolate on *E. coli*, D: The inhibition of HMB 1 isolate on *S.aureus*, E: The inhibition of HMD 4 isolate on *S.aureus*, F: The inhibition of HMD 6 isolate on *S.aureus*. Bar = 1 cm

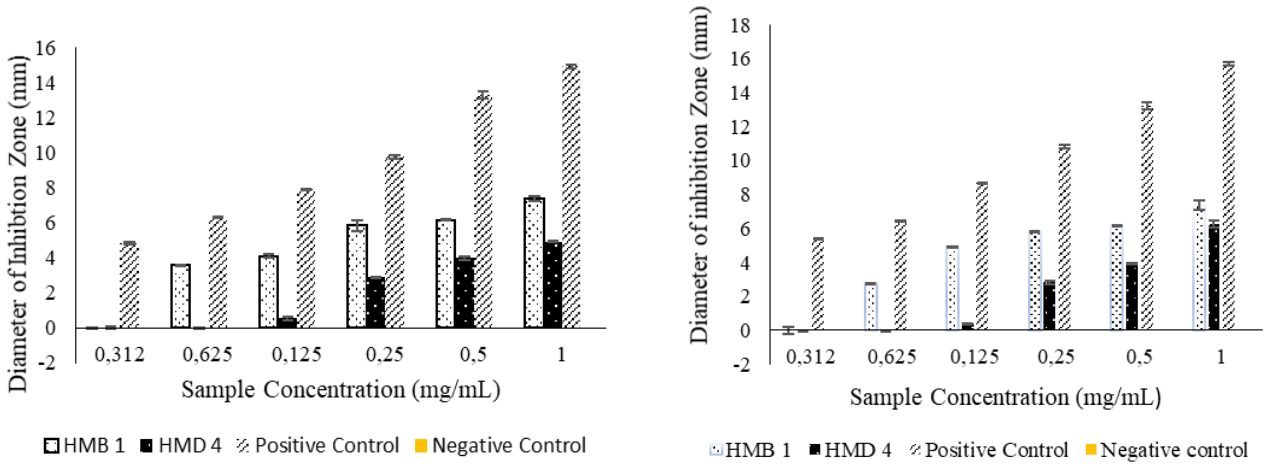

Figure 2. Result of MIC test on bacterial extract against *E. coli* (A) and *S. aureus* (B)

Table 3. Result of GC-MS test for HMB 1 bacterial crude extract

RT (min)	Area (%)	Compounds	Similarity (%)
7.678	1.56	Phenol, 2,4-bis (1,1-dimethyl)	92
8.809	4.09	1-Naphthylbut-1-yn-3-one	90
9.9	2.27	Neophytadiene	99
13.311	2.03	3-benzyl-1,40diaza-2,5-dioxobicyclo	91
13.533	6.32	Pyrollo [1,2-a]pyrazine-1,4-dione,hexahydro-3- (phenylmethyl)	99
15.277	1.48	13-Docosenamide	95

Note: RT: Retention time

Table 4. Result of GC-MS test for HMD 4 bacterial crude extract

RT (min)	Area (%)	Compounds	Similarity (%)
7.678	1.13	Phenol, 2,4-bis (1,1-dimethyl)	92
8.635	1.5	1-Naphthylbut-1-yn-3-one	90
9.909	4.55	Neophytadiene	99
11.969	1.15	Linolenic acid	99
13.368	4.64	Pyrollo [1,2-a]pyrazine-1,4-dione,hexahydro-3- (phenylmethyl)	99
14.141	1.13	1,2-Benzenedicarboxylic acid	91
15.175	1.04	1-Nonadecene	98
15.286	1.77	Oleic acid amide	95
18.835	1.46	Stigmasterol	99

Note: RT: Retention time

Table 5. Percent similarity of the sequences of 16S rRNA gene from isolates HMB 1 and HMD 4

Isolate	Species	Strain	Similarity	Accession number
HMB 1	*Bacillus siamensis*	KCTC 13613	99.91%	KY643639.1
	Bacillus subtilis	168	99.63%	APO19714.1
	Bacillus nakamurai	NRRL B 41091	99.54%	MG846018.1
	Bacillus vallismortis	NRRL B 14890	99.54%	NR 116188.1
	Bacillus subtilis	DSM 10	99.41%	MN077147.1
HMD 4	*Bacillus aryabhattai*	B8W22	100%	NR 118442.1
	Bacillus megaterium	NRBC 15308	99.79%	MK424276.1
	Bacillus flexus	NRBC 15715	99.79%	NR 113800.1
	Bacillus simplex	NRBC 15720	99.08%	NR 115083.1
	Bacillus abyssalis	SCSIO 15042	98.85%	NR 109671.1

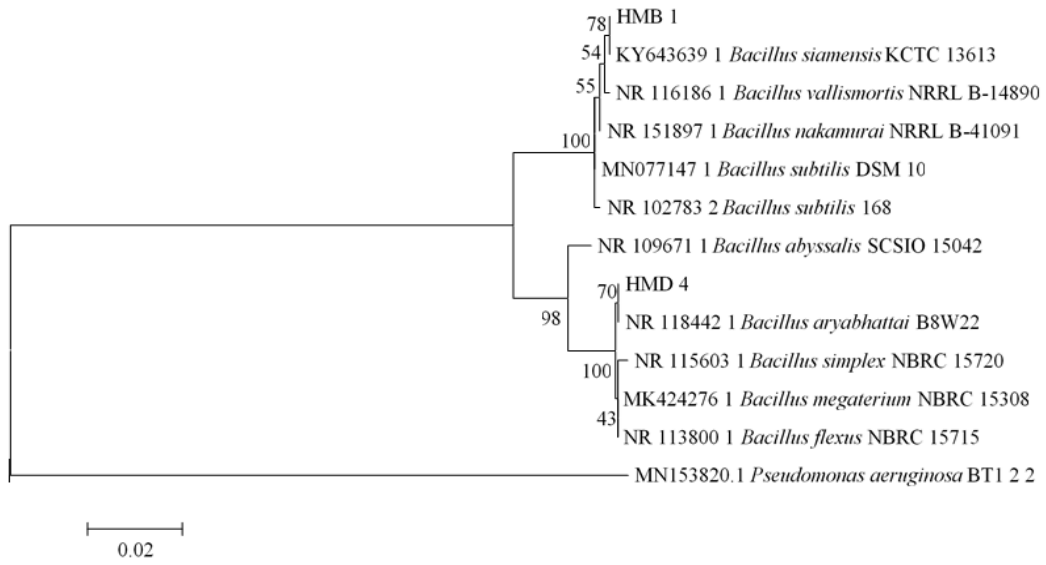

Figure 3. The maximum-likelihood phylogenetic tree of isolates HMB 1 and HMD 4 of endophytic bacteria from *H. multiflora* based on 16S rRNA gene

Discussion

Endophytic bacteria colonize plant tissue but do not have negative effect on the host plant because endophytic bacteria can only live in healthy plants. Endophytic bacteria can be isolated from sterile roots, stems and leaves (El-Tarabily 2003) so that surface sterilization need before isolating endophytic bacteria. About 16.7%, 44% and 38.9% of endophytic bacteria lived in the root, stem and leaves *H. multiflora*, respectively (Table 1). As explained by Lodewyckx et al. (2002) endophytic bacteria are found more in roots, then the leaves or stems. By contrast, the results of the isolation of endophytic bacteria from *H.multiflora*, this can occur because the plants used are epiphytic. The roots used are not the main roots so that the root biomass is more abundant than stems and leaves. In addition, it is also due to bacteria that take the photosynthetic flow needed in all parts of the plant as a source of nutrition (Koomnok et al. 2007). On the other hand, Seo et al. (2010) determined variations in the number of endophytic bacteria depending on the type of plant, soil structure, aged plants, geographical distribution and sampling time. Entry of endophytic bacteria mostly takes place through roots but also through plant parts exposed to direct air such as leaves, flowers, twigs, and cotyledons. Endophytic bacteria can colonize the plant tissues when plant is injured (Siddiqui and Shaukat 2003).

Screening of endophytic bacteria that have potential antimicrobial activity was characterized by the presence of clear zones around the paper disc. A total of 18 isolates tested against *E. coli* and *S. aureus*, of which four isolates were capable of inhibiting the growth of *E. coli* and *S. aureus* (Table 2). Clear zone formed because bacteria are able to produce extracellular antimicrobial compounds (Figure 1) (Kusumawati et al. 2014). These compounds have the ability to control membrane function in test microbial cells like inhibiting various mechanisms including damaging cell walls to occur cell lysis, changing the permeability of cytoplasmic membranes so that cells leak, causing denaturation of cell proteins, inhibiting the functions of enzymes in cells, damaging protein molecules, damaging nucleic acids and inhibiting nucleic acid synthesis (Prescott et al. 2005). Gram-positive and Gram-negative bacteria have different responses to antimicrobial compounds. The difference is due to the cell wall compilers. The cell wall of Gram-positive bacteria consists of 90% peptidoglycan, teichoic acid, and compact cell wall, while Gram-negative are more chemically complex, thinner and less compact. Peptidoglycan makes up only 5 - 20% of the cell wall, and is not the outermost layer, but lies between the plasma membrane and an outer membrane. This outer membrane is similar to the plasma membrane, but is less permeable and composed of lipopolysaccharides (LPS) (Madigan et al. 2015). Inhibition zones are also influenced by the level of sensitivity of the test microorganisms, culture medium, incubation conditions, diffusion rate of antibacterial compounds and concentration of antibacterial compounds (Pasaribu et al. 2013).

Based on the MIC test, chloramphenicol was used as a positive control because it is a broad-spectrum antibiotic that capable to inhibit the growth of Gram-positive and Gram-negative bacteria, while Dimethyl sulfoxide (DMSO) was used as a negative control because it does not give any effect when added (Octaviany et al. 2019). The MIC test results showed that higher the concentration of the sample greater the inhibitory zone produced (Figure 2). The MIC value is determined from the lowest concentration of an extract that can inhibit the growth of microorganisms (Wahi et al. 2011). Zereini (2014) stated that lower the value of antimicrobial concentrations that can inhibit the growth of test microorganisms, the higher the sensitivity of the test microorganisms to the antimicrobial compounds.

Extraction of HMB 1 and HMD 4 isolates using ethyl acetate. Ethyl acetate was chosen as a solvent for extraction of HMB 1 and HMD 4 isolates because it is a semi-polar solvent that is expected to attract polar and nonpolar compounds. Ethyl acetate contains antimicrobial compounds that are able to inhibit or kill microbes higher than other extracts. Fitrial et al. (2008) stated that semi-polar compounds have a higher affinity for interacting with cell walls, so that semi-polar extracts are more effective in inhibiting bacterial growth than polar or non-polar extracts. HMB 1 isolates produce phenol compounds, 1-Naphthylbut-1-yn-3-one, Neophytadiene, 3-benzyl-1,4-diaza-2,5-dioxobicyclo, Pyrrolo [1,2-a] pyrazine-1,4 - dione, 13-Docosenamide, Stigmast-5-en-3-ol. According to Dephour et al. (2012) phenol compounds from *Allium rotundum* capable to inhibit the growth of *Proteus mirabilis*, *Enterobacter cloacae*, *Klebsiella pneumonia*, *Staphylococcus aureus*. Several studies showed bioactivity from bioactive compound: 1-Naphthylbut-1-yn-3-one, Neophytadiene, 3-benzyl-1,4-diaza-2,5-dioxobicyclo, 13-Docosenamide, Stigmast-5-en-3-ol, I-nonadecene , oleic acid amide (Chajjed et al. 2011; Singh et al. 2012; Yousry et al. 2009; Praddhessh et al. 2017; Seanego and Roland (2012); Naragani et al. 2016; Zaher et al. 2015). On the other hand, pyrrolo [1,2-a] pyrazine-1,4-dione obtained from marine bacterial isolates effectively inhibits *S. aureus* (Kiran et al. 2018), more than pyrrolo [1,2-a] pyrazine-1,4 - dione isolated from mangrove land found that *Streptomyces mangrosivoli* isolate can be used as an antioxidant agent (Ser et al. 2015). Linolenic acid compounds are reported to have antioxidant abilities and Stigmasterol potential as antimicrobial and anticancer agents (Subaiyyan et al. 2014). The presence of stigmasterol in crude extracts of HMD 4 isolate from *H. multiflora* leaves is thought to have a relationship with the compounds contained in *H. multiflora* leaf extracts including β-lupeol, α-amyrin, β-amyrin, lupeol acetate, α- amyrin acetate, and β-amyrin acetate and α-amyrin, bauerenol, squalene, lutein, β-sitosterol, stigmasterol from leaves (Ebajo et al. 2015). This is due to the ability of endophytic microbes to produce the same compound as the host by own synthesizing or association with the host (Singh et al. 2017).

To determine the identity of the potential endophytic bacteria i.e. HMB 1 and HMD 4 isolates, PCR analysis of 16S rRNA gene was carried out. The results of identification using the 16S rRNA gene were performed indicated that HMB 1 isolate had the highest similarity

with *Bacillus siamensis* KCTC 13613 (99.91%) and HMD 4 isolate had the highest similarity *with Bacillus aryabatthai* B8W22 (100%). The similarity of BLAST results more than 97% can represent the same species, while 93% to 97% sequence sequences can represent bacterial identities at the genus level (Hagstroom et al. 2000). BLAST showed that all isolates were identified into *Bacillus.* This is relevant to Sun et. al (2013) that the most endophytic bacteria identified as *Pseudomonas*, *Burkholderia*, *Bacillus*, *Paenibacillus.* Jeong et al. (2012) reported that *Bacillus siamensis* KCTC 13613 has antimicrobial activity against *Micrococcus luteus*, *Rhizoctonia solani* and B*otrytis cinerea*. On the other hand, Xu et al. (2018) stated that *Bacillus siamensis* JFL15 is capable to inhibit the growth of *Escherichia coli*, *Edwardsiella tarda*, *Pseudomonas aeruginosa*, *Aeromonas hydrophila*, and *Vibrio* sp. Moreover, *Bacillus siamensis* isolated from endophytic bacteria from Banana (*Musa paradisica* L) produced Gibberellic acid (Ambawade and Pathade 2013) and Indole Acetic Acid (IAA) (Ambawade and Pathade 2018). On the other hand, Bhutani et al. (2018) stated that *B. aryabatthai* isolated from *Vigna radiata* produced plant growth promoters with supplemented tryptophan in medium.

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