Screening of antibacterial activity of seagrass-associated bacteria from the North Java Sea, Indonesia against multidrug-resistant bacteria

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Abstract. Methicillin resistant *Staphylococcus aureus* (MRSA) and multidrug-resistant (MDR) *Escherichia coli* have been reported as a global health issue for several decades. The resistance to common antibiotics has led to the exploration of new potential candidate therapeutic agents to treat the infection. This study was designed to explore the ability of seagrasses-associated bacteria as the sources of antibiotic candidates. Four seagrasses (*Enhalus acoroides*, *Thalassia hemprichii*, *Cymodocea* sp. and *Syringodium* sp.) were collected from the North Java Sea, Indonesia, and carried 70 associated bacteria. Out of all the isolates, only two bacteria exhibited antibacterial activity against MRSA and MDR *E. coli*, namely EED 15 and EED 16. Isolate EED 15 has been classified as *Bacillus flexus* (ACC. number LC123590, 97.72% similarity) and EED 16 as *Streptomyces lienomycini* (ACC. number LC123591, 99.8% similarity).

Key Words: anti-MRSA, anti-*E. coli*, *Bacillus flexus*, *Streptomyces lienomycini*, endophyte, epiphyte.

Introduction. Infections with multidrug-resistant (MDR) bacteria such as methicillin resistant *Staphylococcus aureus* (MRSA) and *Escherichia coli* have been reported as a global health issue (CDC 2017). For many years, these bacteria have been the cause for a high number of hospitalizations in various countries and in several cases it has led to death (Abernethy et al 2015; Cassini et al 2018; Haysom et al 2018; Heiman Marshall et al 2015). The resistance to various antimicrobial agents makes the treatment of MRSA and MDR *E. coli* infections increasingly difficult and multifaceted. Therefore, the exploration of new antimicrobial candidates to treat MRSA and MDR *E. coli* infections is urgently needed.

Indonesia, as a tropical and maritime country, harbors large quantities of marine organisms reported to have antimicrobial activities (Ayuningrum et al 2019; Kristiana et al 2019; Sibero et al 2019). Nevertheless, the ability of associated microorganisms to produce bioactive compounds is related to their host, as several bioactive compounds from marine microorganisms had striking similarities with the metabolites of their hosts (Proksch et al 2002; Radjasa et al 2011). Marine plants such as seagrass contain several prospective antimicrobial compounds such as thalassiolin D, isoscutellarein *7-O-β*-xylopyranoside-2"-O-sulfate and isoscutellarein *7-O-β*-xylopyranoside, from *Thalassia*

hemprichii; phenyl thioketone from *Cymodocea serrulata*; diglyceride ester and asebotin from *Thallasodendron ciliatum* (Gnanambal et al 2015; Hawas 2014; Hawas & Gad El-Mawla 2017; Ibrahim et al 2012). Despite their potential, they are a neglected source of bioactive compounds in Indonesia (Hanif et al 2019).

The lack of reports on bioactive compounds originating from seagrass might be connected to the conservation movement related to the ecological function of this plant. To overcome this, exploration of bioactive compounds from marine-associated microorganisms has been practiced to isolate new candidates from antimicrobial agents to inhibit the pathogenic bacteria from the marine host (Ayuningrum et al 2019; Kristiana et al 2019; Sibero et al 2019). The previous studies on marine organisms were successful in isolating endophytic and epiphytic microorganisms with intense antibacterial activity (Sibero et al 2017; Tarman et al 2013; Ukhty et al 2017). Moreover, several studies reported the ability of endophytic microorganisms to produce similar if not the same bioactive compounds as their hosts (Ludwig-Müller 2015; Newman & Cragg 2015). Seagrass producing antimicrobial compounds allows its endophytic and epiphytic bacteria to produce antimicrobial compounds that could be used against MRSA and MDR *E. coli*. This study was designed to isolate the endophytic and the epiphytic bacteria from seagrass collected from the North Java Sea, to screen the antimicrobial activity against clinical pathogenic MDR bacteria and to identify the isolates that show potential using molecular approach.

Material and Method

Specimen collection and identification. Specimen collection was done at Kemujan Island and Awur Bay, North Java Sea, Indonesia (Figure 1). The seagrasses were collected at a depth of 0 to 3 meters by snorkeling. The seagrasses were put into sterile plastic bags (Whirl-Pak, Nasco, USA) containing 50 mL of sterile seawater and immediately stored in a cooling box.

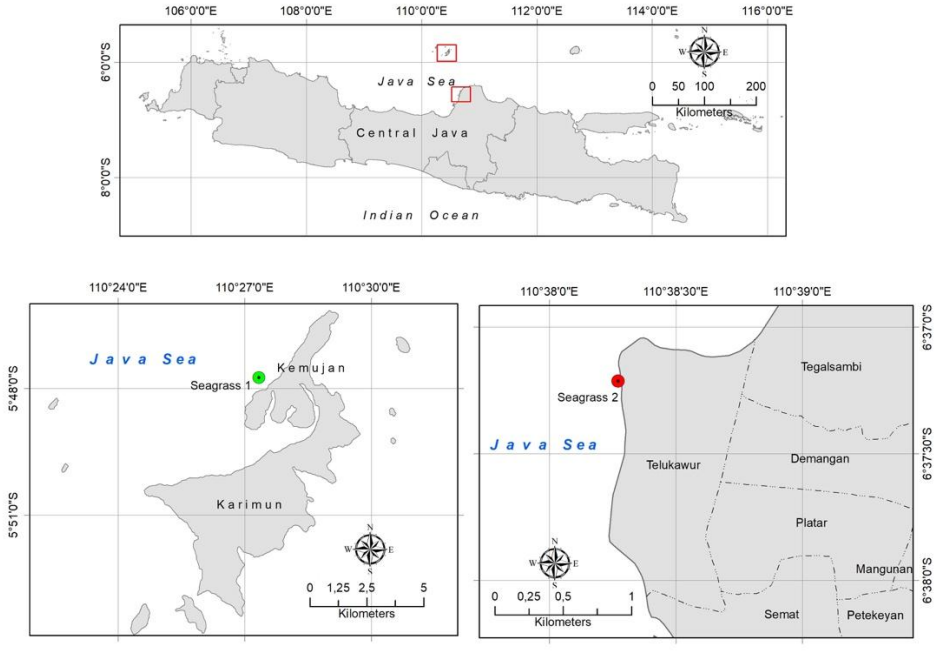

Figure 1. Sampling site of seagrass in Karimunjawa Sea (green) and Awur Bay (red). Source: GeoEye-1 satellite.

Isolation and purification of endophytic and epiphytic bacteria. The green colored leaves of seagrass without signs of discoloration were resized to 1×1 cm. The leaves were washed to isolate the epiphytic bacteria using sterile seawater. The leaves were placed onto Zobell Marine Agar (HiMedia) and Actinomycetes Isolation Agar (Difco™) to isolate

the slow-growing bacteria. The isolation process of endophytic bacteria from seagrass was done in accordance with (Fitri et al 2017), with minor modifications in the surface sterilization step. The surfaces were sterilized to eliminate epiphytic bacteria by rinsing the sample in 70% ethanol for one minute, then washing in sterile seawater and finally washing once again with 70% ethanol. After that, the leaves were cut sagittally to obtain the inner part of the leaves. The inner part was placed onto the isolation media. All media were incubated at room temperature (27°C) until the targeted bacteria cultivation was complete. The successful surface sterilization did not generate any bacterial growth on the control media. This step was performed aseptically to decrease the possibility of environmental contamination.

Antimicrobial susceptibility test for pathogenic bacteria. Clinical pathogenic bacteria were obtained from Dr. Kariadi General Hospital in Semarang, Indonesia. The antibiotic discs were purchased from Oxoid™. Antimicrobial susceptibility testing of MRSA and MDR *E. coli* was performed by disc diffusion in accordance with the criteria of the Clinical and Laboratory Standards Institute (CLSI 2016). MDR *E. coli* was tested for susceptibility to a set of antimicrobial drug discs (BD): kanamycin (30 µg), gentamycin (10 µg), amikacin (30 µg), ampicillin (10 µg), penicillin (10 µg), erythromycin (15 µg), ceftriaxone (30 µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg), vancomycin (30 µg) and ciprofloxacin (5 µg). Additionally, MRSA was tested for susceptibility to a set of antimicrobial drug discs (BD): kanamycin (30 µg), gentamycin (10 µg), amikacin (30 µg), oxacillin (1 µg), erythromycin (15 µg), ceftriaxone (30 µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg), chloramphenicol (30 µg), and ciprofloxacin (5 µg) by use of the disc diffusion method.

Screening of antibacterial activity. The antimicrobial potential of the endophytic and epiphytic bacteria was investigated using antagonistic tests with the overlay method (Radjasa et al 2008; Sibero et al 2019). The bacterial isolates were cultivated on Zobell Agar for five days prior to the screening. MRSA and MDR *E. coli* were cultivated for 24 hours in a Nutrient Broth (NB) medium at 37°C. The bacterial turbidity was adjusted to 0.5 McFarland and mixed with soft nutrient agar (70% agar) medium. This mixture was then poured onto the Zobell agar medium which was previously inoculated with the isolates. The plates were incubated overnight at 37°C. The antimicrobial potential was observed by the formation of inhibition zone/clear zone. A zone of inhibition (ZOI) of 7–11 mm was categorized as *low*, a ZOI of 12–16 mm as *medium*, a ZOI of 17–21 mm as *strong*, and a ZOI≥21 mm as *very strong* antimicrobial activity (Ayuningrum et al 2019). The isolates which performed antibacterial activity were identified using a molecular approach.

Identification of prospective bacteria. For the identification of prospective bacteria, five days old bacteria were used. The bacteria were transferred into a sterile micro tube containing 1 mL of saponin, 100 µL of sterile ddH_2O and left overnight at 4°C (Sibero et al 2019). Extraction of DNA was performed by the Chelex DNA extraction method (de Lamballerie 1992). The PCR reaction (25 mL) consisted of 12.5 µL GoTaq® Master Mix (Promega) (final concentration 1x), 1 µL each of 10 µM concentrations of the forward and reverse primer, nuclease-free water 9.5 µL and 1 µL of extracted DNA template from the seagrass-associated bacteria (final concentration <50 ng). The universal primer 27F 5'-AGAGTTTGATCMTGGCTCAG-3 and 1429R 5'-TACGGYTACCTTGTTACGACTT-3 (Weisburg et al 1991) was used to amplify the 16S rRNA gene. The PCR reaction was conducted in a Thermal Cycler (BIO-RAD) T100 under the following conditions: initial denaturation at 95°C for 3 minutes, followed by 30 cycles at 95°C for 1 minute, 53.9°C for 1 minute, and 72°C for 1 minute and final extension was 72°C for 7 minutes (Ayuningrum et al 2016). The PCR products were examined using agarose 1% gel electrophoresis. The result was visualized with UVIDoc HD5 (UVITEC Cambridge, UK). PCR products were sent to 1st BASE DNA Laboratories (SdnBhd Malaysia) for sequencing process

Phylogenetic analysis. Basic Local Alignment Search Tool (BLAST) (www.ncbi.nlm.nih.gov) was used to determine the closest related bacteria species with active isolates. A maximum likelihood (ML) phylogenetic tree of these isolates was constructed using Molecular Evolutionary Genetics Analysis (MEGA) running software version 7. A GTR+G+I model with 1,000 bootstrap replications was used to construct a phylogenetic tree of active isolates. The obtained sequences have been deposited to the DNA Data Bank of Japan (DDBJ) with the accession number at www.ddbj.nig.ac.jp.

Results and Discussion

Seagrasses and the abundance of their associated bacteria. Seagrasses are defined as a group of flowering plants found in marine environments. This plant's complete life cycle is under seawater (Kuo & McComb 1998). In Indonesia, several seagrass species have been found, such as *Cymodocea rotundata*, *C. serrulata*, *Enhalus acoroides*, *Halodule uninervis*, *T. hemprichii*, *Syringodium isoetifolium* (Ambo-Rappe et al 2013; Riniatsih et al 2019; Riniatsih et al 2018). In this study, four of these species were examined, namely *E. acoroides* and *T. hemprichii* from Kemujan Island, and *Cymodocea* sp. and *Syringodium* sp. from Awur Bay, North Java Sea (Figure 2). These locations have been known as the natural habitat of seagrasses. Prasetya et al (2017) reported the percentage cover of *E. acoroides* to be 5.48–7.27% and *T. hemprichii* to be 6.95–18.02% in Kemujan Island. Furthermore, a previous study by Riniatsih et al (2019) reported on the existence of *Cymodocea* sp. and *Syringodium* sp. in Awur Bay. These seagrasses host prospective bacteria, according to the numerous reports on antimicrobial compounds produced by these species (Gnanambal et al 2015; Sathyanathan et al 2016; Hawas 2014; Hawas & Gad El-Mawla 2017; Ibrahim et al 2012; Palanisamy et al 2014). Furthermore, endophytic microorganisms were reported to have the capability of producing similar bioactive compounds as their host, including antibacterial (Ludwig-Müller 2015; Newman & Cragg 2015; Singh et al 2017). Therefore, it was expected that the endophytic and epiphytic bacteria from these seagrasses had antibacterial potency to inhibit the MRSA and MDR *E. coli*.

Seagrass-associated bacteria in this study were divided into endophytic and epiphytic bacteria. Endophytic bacteria are defined as the bacteria living within the plant tissue without causing any disease or illness to the host, whereas epiphytic bacteria live on the surface of their host (Ludwig-Müller 2015; Newman & Cragg 2015; Singh et al 2017). The isolates were tested against two MDR pathogenic bacteria that resulted in two active isolates as shown in Figure 3.

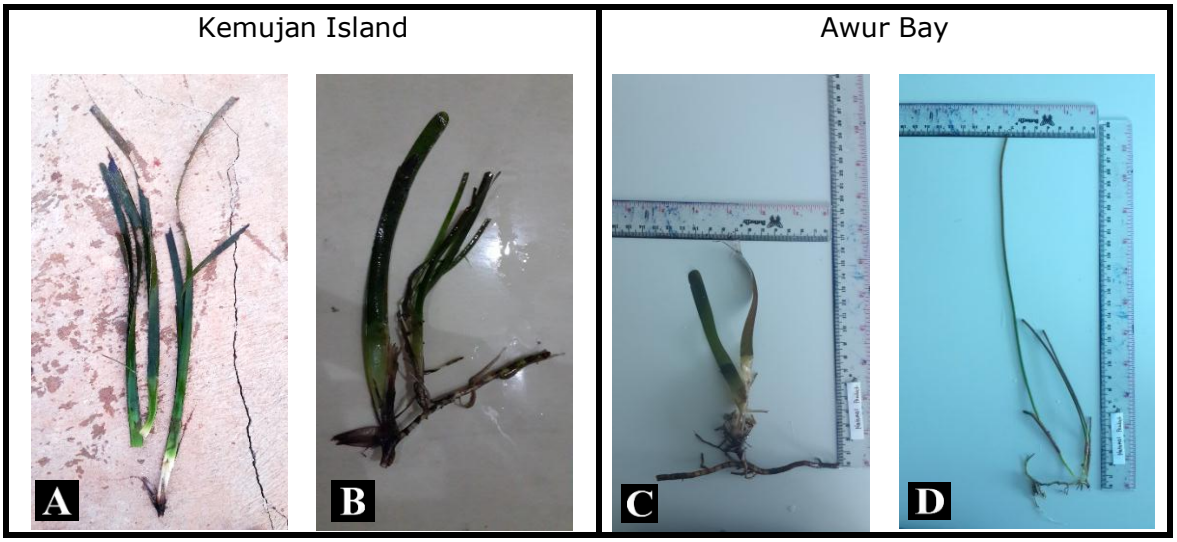

Figure 2. Morphology of seagrass collections from sampling sites. A) *Enhalus acoroides*, B) *Thalassia hemprichii*, C) *Cymodocea* sp., D) *Syringodium* sp.

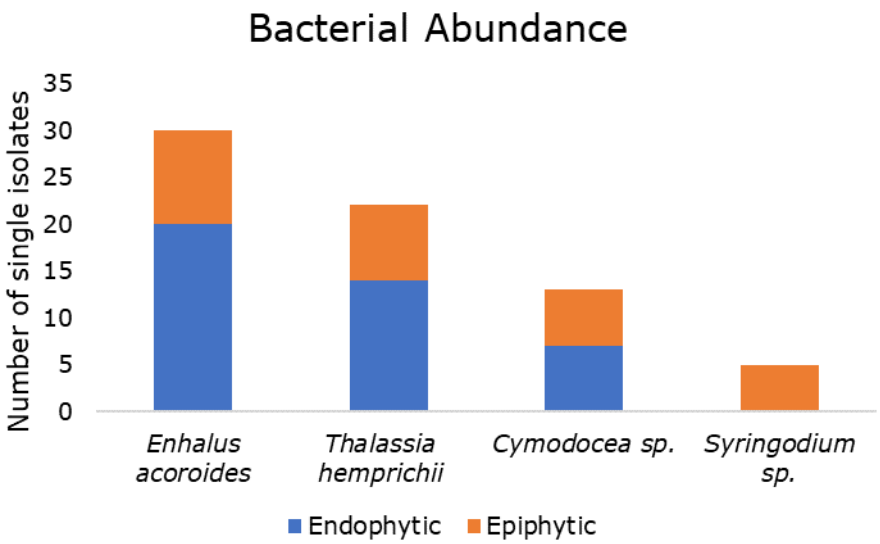

Figure 3. Bacterial abundance of the seagrass samples.

Figure 3 shows that *E. acoroides* had the most abundance-associated bacteria with 30 isolates, followed by *T. hemprichii* with 22 isolates, *Cymodocea* sp. with 13 isolates and the lowest abundance was found in *Syringodium* sp. with 5 isolates. The microbial abundance is affected by various environmental factors such as salinity, temperature, ocean acidification and nutrients in the water (Currie et al 2017; Hou et al 2017; Sjöstedt et al 2014). All isolates were tested against MRSA and MDR *E. coli* to screen the potential candidate as antimicrobial compounds producers.

Screening of antimicrobial activity. MDR bacteria is a category of bacteria that have developed a resistance to several classes of antibiotics (Magiorakos et al 2011). In this study, we performed the susceptibility test to confirm the resistance status of MRSA and MDR *E. coli* provided by Dr. Kariadi General Hospital in Semarang as the clinical pathogen. The results of the antimicrobial susceptibility test are shown in Table 1.

Based on antimicrobial drugs tested against our isolates, *E. coli* strains proved resistant to ampicillin, penicillin, erythromycin, ceftriaxone, trimethoprim-sulfamethoxazole, vancomycin and ciprofloxacin. *S. aureus* also showed resistance to kanamycin, gentamycin, amikacin, oxacillin, ceftriaxone, chloramphenicol and ciprofloxacin. As such, these clinical pathogens are classified as MDR bacteria according to CLSI (2016). The seagrass-associated bacteria were tested to inhibit the growth of these clinical pathogenic MDR bacteria. Figure 4 and Table 2 present the results of the antibacterial screening.

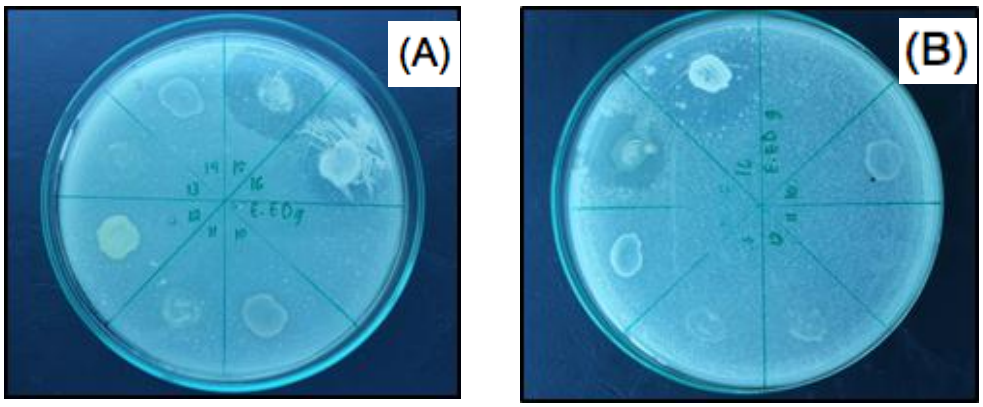

Figure 4. Inhibition zone seagrass-associated bacteria against (A) *Escherichia coli* and (B) *Staphylococcus aureus*.

Table 1

Antibiotic susceptibility profile of the pathogens

No	*Bacteria*	*K*	*GM*	*AN*	*AM*	*OX*	*P*	*E*	*CRO*	*SXT*	*VA*	*C*	*CIP*
Class of antibiotics		*Aminoglycosides*			*β-lactamase*			*Macrolide*	*Cephalosporin*	*Sulphonamides*	*Glycopeptide*	*Phenicols*	*Quinolon*
1	*E. coli*	I	S	S	R	-	R	R	R	R	R	I	R
2	*S. aureus*	R	R	R		R	R	I	R	S	-	R	R

K: Kanamycin; GM: Gentamycin; AN: Amikacin; AM: Ampicillin; OX: Oxacillin; P: Penicillin; E: Erythromycin; CRO: Ceftriaxone; SXT: Trimethoprim-Sulfamethoxazole; VA: Vancomycin; C: Chloramphenicol; CIP: Ciprofloxacin.

Table 2

The results of antimicrobial screening against clinical pathogenic MDR bacteria

Seagrass	*Number of isolates*	*Active candidate*			
		E. coli		*MRSA*	
		Endo	*Epi*	*Endo*	*Epi*
E. acoroides	30	EED 15 EED 16	-	EED 15 EED 16	-
T. hemprichii	22	-	-	-	-
Cymodocea sp.	13	-	-	-	-
Syringodium sp.	5	-	-	-	-

Endo: Endophyte; Epi: Epiphyte; EED: *Enhalus* endophyte; -: no active candidate.

Results of the antimicrobial screening showed that there were only two active isolates among the 70 seagrasses-associated bacteria. These were EED 15 and EED 16 (Table 2). Isolates EED 15 and 16 as endophytes were isolated from *E. acoroides* and using the overlay method showed strong antibacterial activity against both MRSA and MDR *E. coli* (Figure 4). Fitri et al (2017) reported a similar result to that using the overlay method, endophytic bacteria of *E. acoroides* collected from East Nusa Tenggara, Indonesia had more potential to inhibit MRSA and *Bacillus cereus* than endophytic bacteria from *C. rotundata* and *T. hemprichii*. The overlay method is a common technique to screen antimicrobial potential of microbes from marine environments (Asagabaldan et al 2016; Ayuningrum et al 2019; Kristiana et al 2019; Sibero et al 2019). An environmental stress was induced to the isolates by the addition of a pathogen-agar layer on top of the colony. The potential isolate produced antimicrobial compounds, and then secreted them into the environment which then penetrated into the pathogen-agar layer. The growth of pathogens that were exposed to antimicrobial compounds from the potential isolate are inhibited as indicated by the clear zone (Balouiri et al 2015).

Bacterial identification. As prospective isolates, EED 15 and EED 16 were identified using a molecular approach to understand its species. The results of bacterial BLAST homology are shown in Table 3, while the phylogenetic tree is presented in Figure 5.

Table 3

Result of BLAST homology of prospective isolates

Isolate	*Identical species*	*% Similarity*	*Accession number**
EED 15	*Bacillus flexus* BTNGP3 MK610727	97.72%	LC123590
EED 16	*Streptomyces lienomycini* NBRC15425 NR112464	99.98%	LC123591

*has been submitted to the DNA Data Bank of Japan (DDBJ) at www.ddbj.nig.ac.jp for the accession numbers.

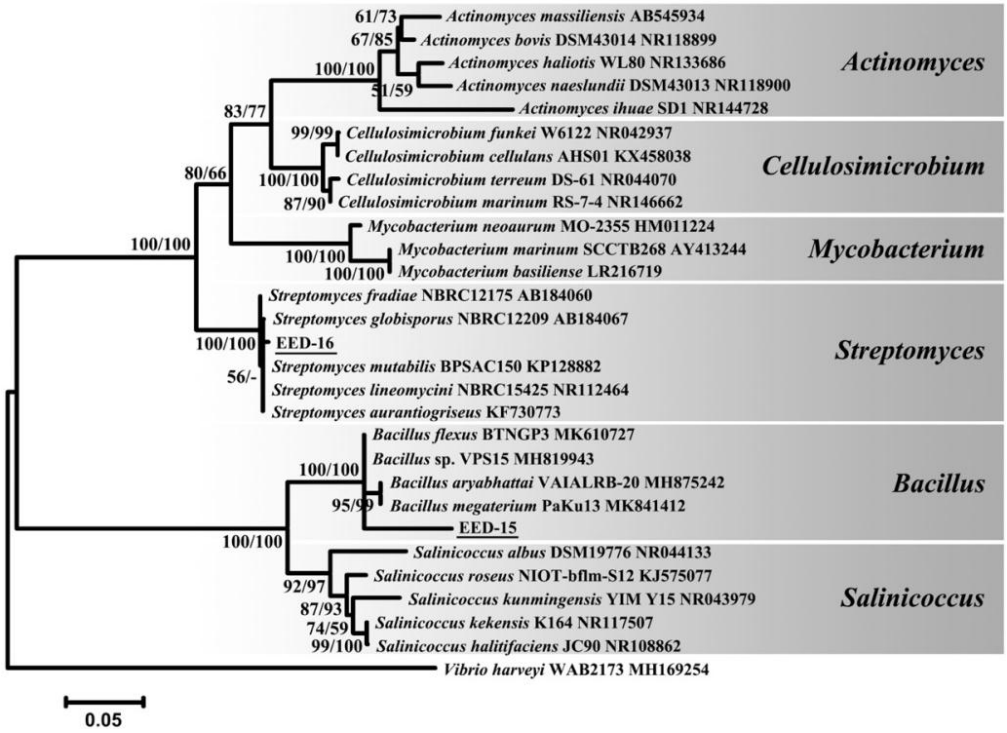

Figure 5. A maximum likelihood (ML) phylogenetic tree based on 16S rRNA genes of prospective isolates. Numbers at nodes represent bootstrap values (NJ/ML). The bootstrap values less than 50% are not shown.

Phylogenetic inference of 16S rRNA gene showed that strain EED-16 formed tight clade together with several sequence data of *Streptomyces* sp., whereas EED-15 clustered in *Bacillus* clade. In more detail, the results of the BLAST homology exhibited that EED 15 was genetically identical with *B*. *flexus* (acc. number BTNGP3 MK610727) and EED 16 to *S. lienomycini* (acc. number NBRC15425 NR112464), with a homology of 97.72% and 99.98%, respectively. Studies of seagrass-associated bacteria are quite rare and there are only few reports available. Previous studies on this subject were focused on the fungal endophyte rather than the bacterial endophyte (Mata & Cebrian 2013; Raja et al 2016; Supaphon et al 2017; Thirunavukkarasu & Pon 2016). Interestingly, Fitri et al (2017) also found *B*. *flexus* as the endophytic bacteria of *E*. *acoroides* from East Nusa Tenggara, Indonesia together with *Bacillus megaterium*. In addition, they also stated that *B*. *flexus* showed antibacterial activity against MRSA and *B*. *subtilis*. Furthermore, *Streptomyces* sp. is an actinobacteria which previously has been isolated from marine environment as endophyte (El-Gendy et al 2018; Rajivgandhi et al 2018). The genus is commonly reported as a productive source of secondary metabolites with various biological activities. In 2018 there were several species of *Streptomyces* isolated from the marine environment in Iran (Norouzi et al 2018). Their study found three isolates that performed antibacterial activities against MRSA and Vancomycin-Resistant *S. aureus* (VRSA). Prior to that, El-Naggar & Hamouda (2016) found that *S*. *lienomycini* has antimicrobial activity against MDR *Pseudomonas aeruginosa*. These reports support the results of our study in that *S*. *lienomycini* from *E*. *acoroides* from Kemujan Island was also recognized as a potential source of new candidate for antibiotics for the treatment of MRSA and MDR *E*. *coli* infections. Finally, follow-up research into bioactive culture production, compounds isolation and advanced bioassays are suggested.

Conclusions. There were 70 seagrass-associated bacteria consisting of 41 endophytes and 29 epiphytes isolated from four Indonesia's seagrasses. *E*. *acoroides* gave the highest bacterial abundance (30 isolates), followed by *T*. *hemprichii* (22 isolates), *Cymodocea* sp. (13 isolates) and *Syringodium* sp. gave the lowest bacterial abundance (5 isolates). Among all isolates, there were only two bacteria (EED 15 and EED 16) that inhibited the growth of MRSA and MDR *E*. *coli*. According to molecular identification, EED 15 was identified as *B*. *flexus* (Acc. number LC123590, 97.72% similarity) and EED as *S. lienomycini* (Acc. number LC123591, 9.98% similarity).

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