Original article

Uji lanjutan potensi bakteri simbion lamun *Enhalus acoroides* dan *Thalassia hemprichii* sebagai antibakteri terhadap agensia penyebab penyakit Vibriosis

Advanced test on potential of bacteria symbionts seagrass Enhalus acoroides *and* Thalassia hemprichii *as antibacterial towards disease cause agents Vibriosis*

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Abstract

This study is the further study on isolation and identification of bacterial symbionts and Enhalus acoroides and Thalassia hemprichii. The results showed that the activity of bacterial symbionts seagrass Enhalus acoroides and Thalassia hemprichii acquired seven bacterial isolates which were able to inhibit Vibrio harveyimasing - each isolate consisted of 5 Thalassia hemprichii isolates and 2 Enhalus isolates. The results of the study showed that the activity of bacterial symbionts seagrass Enhalus acoroides and Thalassia hemprichii acquired 7 bacteria isolates to inhibit Vibrio. There were 4 isolates that had bactericidal activities. Two bacterial symbionts that had the highest inhibitory zones were identified, namely Th 12 isolate, type of bacteria Bacillus macerans, meanwhile Eh 10 isolate was Micrococcus varians bacteria. The results showed that the inhibitory zone Eh 9 isolate (3.87 ± 0.25 mm) was higher than Th 20 isolate (3.14 ± 0.18 mm). Based on the results of biochemical identification of bacteria known that Eh 9 was kind of Brevibacterium sp ., while the Th 20 isolate was a type of bacteria Bacillus pumilus. The results concluded that the bacterial symbionts that comes from Enhalus acoroides and Thalassia hemprichii can serve as an antibacterial of Vibrio harveyi.

Keywords: Seagrass, *Enhalus acoroides*, *Thalassia hemprichii*, *symbiont bacteria*, antibacterials, *Vibrio harveyi, biochemistry test*

Introduction

Various studies have been conducted to find a method of prevention and dealing with disease on shrimp, and one of the attacking diseases is *vibriosis*. *Vibrio harveyi* is one of the bacteria causing desease *vibriosis* (Riniatsih and Setyati, 2009). One way to overcome this vibriosis disease is by using antibiotic or antibacterial. Antibiotic substances can inhibit and even kill microorganisms.

Pathogen but the use of antibiotics had indeed caused new problems because it is not environmentally friendly. Antibiotic substances can increase the resistance of pests that want to be addressed, so it does not work or the dose used will continue to increase (Soemardiharjo et al., 1999). If the such condition constantly happens, it will lead to pressure on the ecological condition of the environment. Development of new antibiotics is one important solution in the treatment of drug-resistant bacteria that were previously incompatible with existing antibiotics. During this time antibiotics were taken from terrestrial organisms, exploiting sea organisms for the pharmaceutical field was still rare. On the other hand, it is known that secondary metabolism of some marine flora and fauna can be used as drugs. This is because the marine flora and fauna can produce chemical compounds that one of its functions is as an anti-bacterial (Pringgenies et al, 2006 and Pringgenies, 2011).

Seagrass is one of a group of marine flora and higher level flowering plants (*Angiospermae*) and has adapted to live immersely in shallow sea water with sand and mud. Seagrass has rizhoma, leaves and true roots (Nontji, 1987) as well as plants on land.

The research on bacterial symbionts with seagrass has been done before by Ravikumar et al. (2010) with the results the potential of bioactive of bacteria seagrass as antibacterial agent of bacterial human pathogens which showed that the antibacterial activity of the kind of seagrass *Cymodocea serrulata* which inhibits the growth of pathogenic bacteria *Staphylococcus aureus*, *Klebsiella sp., Pseudomonas aeruginosa*, *Streptococcus pneumoniae* and *Streptococcus aeruginosa*. A work of Riniatsih Setyati (2009) concerning the Bioactivity Extract and Powdered Seagrass *Enhalus acoroides* and *Thalassia acoroides* on *Vibrio alginolyticus* and *Vibrio harveyii* stated that extracts and powders of simplicia *E. acoroides* showed no bioactivity against *V. alginolyticus* and *V. harveyii*. *T. hemprichii* extract showed bioactivity against *V. alginolyticus*. Research on the potential of bacterial symbionts seagrass *Enhalus acoroides* and *Thalassia hemprichii* as antibacterial against bacteria Vibrio harveyi had been done. The results showed that the results showed that the activity of bacterial symbionts seagrass *Enhalus acoroides* and *Thalassia hemprichii* has seven bacterial isolates that were able to inhibit *Vibrio*. There were 4 isolates that have bactericidal activity. Two bacterial symbionts which had the highest inhibitory zone type of bacterium *Bacillus macerans* and *Micrococcus variance* (Kurniawan et al, 2015). There are two remaining isolates of bacteria that have bactericidal activity and no infomation about them. Based on this, the goal of further research is aiming to find out further information from the two isolates which have bactericidal activity and identification of the bacteria.

Materials and methods

The materials of the study were bacterial symbionts of seagrass *Enhalus acoroides* and *Thalassia hemprichii* taken from Awur Gulf waters, Jepara. The bacteria test used were *Vibrio harveyi* which cause disease on shrimp.

Sampling seagrass acoroides E. and T. hemprichiiv and isolation symbiont bacteria E. acoroides and T. hemprichii had been done ((Kurniawan et al, 2015).

Antibacterial Activity Test Seagrass symbiont bacteria against V. Harveyi

Antibacterial activity test was conducted between the bacterial symbionts of the outcome of the screening of bacteria with bacteria *V. harveyi* by using disk diffusion method (Radjasa et al., 2007). A total of 100 mL of *V. harveyi* bacteria that had been cultured for 24-48 hours mediated liquid spread on a petri dish containing media Zobell 2216E. Symbiont bacteria *E. acoroides* and *T. hemprichii* cultured for 24-48 hours at 2216E Zobell liquid media. Paper disc diameter of 8 mm was placed on a petri dish that had been deployed with the bacterium *V. harveyi*. Paper disks were impregnated as much as 30 mL of bacterial symbionts *E. acoroides* and *T. hemprichii*. Then, the petri dish was covered with plastic wrap to prevent contamination and incubated upside down for 24-48 hours at room temperature. The tests were performed in two repetitions and the inhibition zone was calculated in units of mm.

The antibacterial activity could be seen by observing the inhibition zone formed around the paper disc. The antibacterial activity would be stated positive if the the inhibitory zone was formed in the form of a clear zone around the paper disc, and the antibacterial activity would be declared negative if there was no clear zone formed. Measurement of the inhibition zones conducted at 48 hours.

Identification of Bacteria in Morphology and Biochemistry

The symbiont bacteria isolates seagrass *E. acoroides* and *T. hemprichii* that can inhibit the growth of bacteria *V. harveyi* were later identified by species biologically using biochemical tests. The method used in biochemical tests by Cowan and Steels (1974) and Bergey's (2005). There are several tests in the identification of bacterial symbiont type of seagrass: Bacteria Morphology Observation, Gram painting, motility test, oxidase test, Catalase test, LEIFSON Hugh test (O / F), Indol test, and nitrate reduction test. For more details, then the lines of inquiry indicated on the following Fig.1.

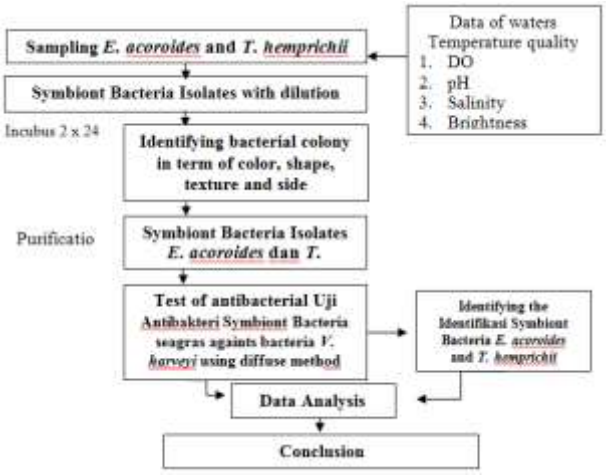

Figure 1. Schematic Flow Research Isolation and Identification of Bacteria symbiont Seagrass Enhalus acoroides and Thalassia hemprichii as antibacterial againts Vibrio harveyi

Results and discussions

Antibacterial Activity Test of *Enhalus acoroides* and *Thalassia hemprichii* symbiont bacteria against *Vibrio harveyi*

From this test was obtained 7 isolates possed antibacterial activity capable of inhibiting the growth of bacteria *V. harveyi*. This was demonstrated by the emergence of clear inhibition zone surrounding the paper disc that can be seen in Figure 2. The results of the measurement of inhibitory zone are presented in Table 1.

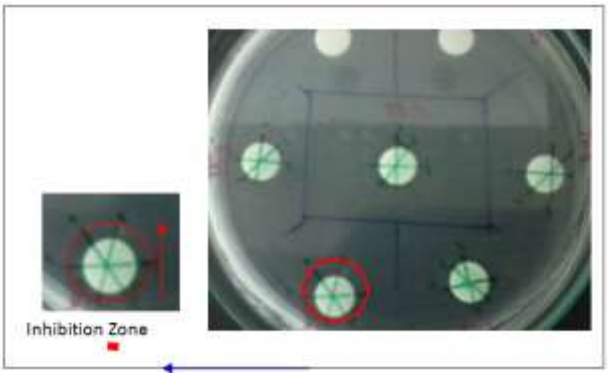

Figure 2. Antibacterial activity of *E. acoroides* dan *T.hemprichii* againts *Vibrio harveyi*

Table 1. Diameter of Inhibition Zone (mm) at Antibacterial activity of bacteria test

Code	Diameter of Inhibition Zone (mm)	Activity
Th 4	3,68 ± 1,65	Static
Th 6	3,06 ± 0,21	Static
Th 9	2,86 ± 1,70	Static
Th 12*	3,24 ± 0,55	Sidal
Eh 10*	4,57 ± 0,74	Sidal
Th 20	3,14 ± 0,18	Sidal
Eh 9	3,87 ± 0,25	Sidal

Note: Average ± Standard deviation (n=2)
(*): Identified bacteria isolates

Based on the results of measurements shown in Table 1, isolates Eh 9 has a higher inhibitory zone size (3.87 ± 0.25 mm) compared to isolate Th (3.14 ± 0,18mm).

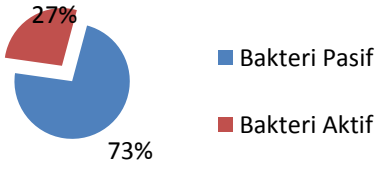

Figure 3. Graph of Antibacterial Activity symbiont bacteria *Enhalus acoroides* dan *Thalassia hemprichii* against Vibrio harveyi

Percentage of bacterial symbionts *E. acoroides* and *T. hemprichii* that can inhibit V. harveyi bacteria by 27% (7 of 26 total bacteria). Meanwhile, the percentage of bacterial symbionts

that can not inhibit the bacterium *V. harveyi* by 73% (19 of 26 bacteria) (Fig. 4).

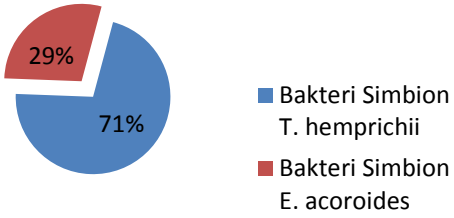

Figure 4. Graph of the total comparison of active bacterial symbionts seagrass on antibacterial activity

Total active bacterial symbionts are 27% (7 of 26 total bacteria) consisted of 71% (5 isolates) bacterial symbionts seagrass *T. hemprichiidan* and 29% (2 isolates) symbionts bacteria E. acoroides seagrass.

Identification on Bacteria in Morphology and Biochemistry

The results of biochemical tests on identifiction the type of bacteria are presented in Table 2.

Table 2. Test Results of Biochemical Identification on Bacteria Biofilm Using Method Cowan and Steels (1974) and Bergey's (2005)

Test of Biochemica	Code of Isolate	
	Th 20	Eh 9
Shape	Blade	Blade
Gram	+	+
Motility	+	-
Length of cell > 3 mm	-	-
Position and spore shape	VX	-
Spore	+	-
Growth on 50^0	+	X
Growth with 10% NaCl	+	X
An aerobic	-	-
Aerobic	+	+
Acid from ASS medium:		
- Glucose	+	X
- Calaboose	+	X
- Galactose	+	X
- Mannose	+	X
- Melibiose	+	X
- Rafinose	-	X
- Salicin	+	X
- Xylose	-	X
OPNG	+	X
Utilization of Citrate	-	X
Urease	-	X
Indol	-	-
VP	+	-
Nitrat reduced	-	-
Casein hydrolysis	+	X
Starch hydrolysis	-	X
Oxidize	+	-
Catalase	+	+
Pigmen	-	+ (Yellow)
Gelatin	X	+

Note : VX: Central/Oval, X: Not in testing

The identification results showed that isolates Eh 9 is a bacterium Brevibacterium sp and isolates Th 20 is a type of bacteria Bacillus pumilus.

The test results of antibacterial activity between the bacterial symbionts *seagrass E. acoroides* and *T. hemprichii* with *V. harveyi* showed that of 26 isolates only 7 bacterial isolates capable of forming inhibition zones. Bacterial isolates that managed to form inhibition zone consists of two seagrass symbiont bacteria *E. Acoroides* dan 5 isolates bacterial symbionts seagrass *T. hemprichii*. Based on the number of bacterial symbionts, the bacterial symbionts of seagrass *T. hemprichii* were better than the bacterial symbionts of seagrass *E. Acoroides* IN inhibiting bacterial *E. V. harveyi*.

At least four bacterial isolates were able to inhibit *V. harveyi* allegedly because *V. harveyi* is a gram-negative bacterium which has thicker composition and wall structure. It has three layers wall cell structure. Gram negative bacteria have a thin cell skin structure (10-15 nm) with triple layers. The peptidoglycan is on rigid layer inside and around 10% of the dry weight, the outer membrane cell wall layer containing high lipids (11-22%) (Madigan and Martinko, 2003). Pelczar and Chan (1988) said outer membrane serves as molecule filter and an asymmetric membrane consists of a layer of phospholipids, lipopoly saccharide, lipoprotein and protein, so outside molecule can not enter easily. In addition, the Gram-negative bacteria have endotoxins in form of poly saccharide which is in certain circumstances be toxic, able to issue molecule which wants to enter the cell.

The disappearance of the inhibition zone is not only caused by the characteristics of test

bacteria used but also influenced by several factors. Some of the factors that affect the appearance of inhibition zone depends on the diffusion of anti-microbial materials into the medium and its interaction with test microorganisms, the number of microorganisms that are used, the speed of growth of microorganisms tested and the sensitivity of microorganisms to anti-microbial material being tested (cappuccino and Sherman, 1996).

The biggest inhibition zone which appeared on the antibacterial activity test for bacterial symbionts were isolates of *E. acoroides* Eh 9 which has a higher inhibitory zone size (3.87 ± 0.25 mm) compared to isolate Th (3.14 ± 0,18mm) with bactericidal activity. Meanwhile, Th 4 has a greater inhibitory zone (3.68 ± 1.65 mm), but the type of antibacterial activity is bacteriostatic. According to Madigan et al. (2011), bacteriostatic agents inhibit protein synthesis by binding to ribosome of an organism for a while. The bonds are not so strong that when the concentration and stability declined, the antimicrobial agent will release the ribosome so that the bacteria can grow again. This differs from the mechanism bactericidal agent which works with strong binding target cells, does not release the bonds anymore and the cells of microorganisms will be killed. The test of antibacterial activity spectrum coverage of this research was limited, because the antibacterial were only slightly effective against bacteria (bacterial isolates 7 of 26 bacteria).

The study using symbiotic bacteria as antibacterial has been implemented before. It is found from the study that of 54 isolates tested seagrass antibacterial activity, there are three bacterial isolates extract which have the ability to inhibit test bacteria *S. aureus*, namely *E. acoroides* (Lisdayanti (2013). Praptiananda (2011) declares that there is only one isolate bacteria from 9 isolates bacterial symbiosis with plants *Enhalus sp.* seagrass which has antibacterial activity against *Mycobacterium tuberculosis*. The results of these studies showed that the bacterial symbiont of seagrass is potential as an anti-bacterial material.

The mechanism of antibacterial inhibition against bacterial growth may be in form of damaging cell wall resulting in lyses or inhibition synthetic cell walls, changing the permeability of the cytoplasmic membrane, causing the release of groceries through the cell wall, protein denaturation of cells and destruction of metabolic systems in the cell by inhibiting the work of intracellular enzymes (Pelczar and Reid, 1972).

The ability of bacterial isolates associated with seagrass in inhibiting microbial growth targets is a form of antagonistic activity undertaken by producing antimicrobial compounds. The biosynthesis of antimicrobial compounds plays an important role in the process of adhesion, colonization of the target until the competition in getting a room and nutrition with other microbes (Romanengko et al., 2008).

These results indicate that the bacterial symbiont derived from *E. acoroides* seagrass has better antibacterial activity than the bacterial symbiont *T. hemprichii* seagrass againts *V. harveyi*. It can be seen from the number of bacteria that actively inhibits bacterial growth *V. harveyi*.

The results of the bacterial symbionts seagrass, namely isolate Eh 9 is kind of *Brevibacterium sp*, these bacteria have not been identified to species. But it was already known that the type of bacteria have antibacterial activity. Matthew, 2006, said that the genus Brevibacterium bacteria belonging to the organism low level pathogenic. Generally it takes a long time in the host's immunological defenses cause significant infection. Bacteria *Brevibacterieae*, especially *Brevibacterium linens* and *B. casei* has been known to be involved in contributing to the aroma of various cheeses Rattray et al (1999). While Pringgenies et al (2016b) states that the type of bacterial symbionts found in gastropods *Melo melo* type is the type of bacterial strain *Brevibacterium celere* KMM 3637 with 89% homology which have antibacterial activity.

On the other hand it is known that bacteria of the genus *Brevibacterium* also known to have anti-oxidant content. The results showed that the components of the algicidal in bacteria genus *Brevibacterium* may serve to degrade the toxins contained in cells. It was alleged that the content of (2-isobutoxyphenyl) amine of actinomycete bacteria genus *Brevibacterium* may be a candidate for controlling poison contained in *Dinoflagellates* (Xinli An, 2015).

The results of the bacterial symbionts seagrass, namely isolate Th 20 is a kind of *Bacillus pumilus*. Bacteria types Baccillus is very tough species, the bacteria forming spores. This type of species is one kind of bacteria represented in samples collected from the surface of the spacecraft. (La Duc et al, 2003 ;. 2004; Puleo et al., 1977). The bacteria are able to survive at the unfavorable environment in space. There is a type of bacteria *Bacillus subtilis* derived from waste

symbionts mangrove potential as a liquid fermentation for compost (Pringgenies, 2016 a).

Meanwhile the types of bacteria *Bacillus pumilus* was found to be the most dominant species both in the aerobic bacteria that form spores (La Duc et al., 2004). *B. pumilus* isolates showed resistance to H2O2 (Kempf et al., 2005) and is thus considered problematic because H2O2 microbial recommended for use in the process bio reduction spacecraft components. This type of bacteria Bacillus pumilus has Gram-positive, aerobic, spore-forming bacillus in character which is commonly found in soil (FG Priest, 1993), but is now found in seagrass. A strain of *B. pumilus* was isolated from black tiger shrimp (*Penaeus monodon*) were found to have a high salt tolerance and inhibit the growth of marine pathogens, including *Vibrio alginolyticus*, when cultured together (Hill, et al, 2009).

From the results of the study, it is concluded that bacterial symbionts of plants seagrass *Enhalus acoroides* and *Thalassia hemprichii* are potential as anti-bacterial. Its Isolate symbionts bacteria have inhibitory zone against *Vibrio harveyi*, namelly isolates Uh 9 (3.87 ± 0.25 mm) and isolates Th 20 (3.14 ± 0.18 mm). Based on the results of biochemical identification of bacteria, it is found that Eh 9 is the kind of *Brevibacterium sp.,* and isolates Th 20 is the kind of bacteria Bacillus pumilus.

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