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RESEARCH ARTICLE

Antibacterial activity of Actinomycetes symbiont with seaweeds: a prosperous agent of animal antibacterial

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ABSTRACT

Circulation of synthetic antibacterial chemicals which is used continuously can cause resistance to bacteria. Alternative synthetic antibiotics research then comes up as an urgent need. Recently, the discovery of new antibacterial agents is looking for Actinomycetes. Actinomycetes are one of the active marine bacteria which produce secondary metabolites that could be used as antibacterial. The objective of this study was to evaluate the antibacterial activities of Actinomycetes symbiont with *Halimeda macroloba, Gelidiella acerosa,* and *Turbinaria ornata* in Pangandaran beach, Indonesia. A total of 41 Actinomycetes were isolated from 3 species of seaweed. Rep PCR amplification method was used to characterize and identify the relationship between samples. Primer BOX A1R (5'-CTACGGCAAGGCGACGCTGACGCTGACG-3'), 27F (5'-AGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') was used in amplification 16s DNA. Six isolates from the representative cluster dendogram were selected for secondary metabolites extraction using ethyl acetate solvent, meanwhile agar diffusion method was used as Antibacterial activity assay using *E. coli, Proteus* sp., and *Enterobacter* sp. as the bacterial test. The results showed that the activity of secondary metabolite extracts of Actinomycetes symbiont with *Halimeda macroloba* presented the highest inhibition zone on 3 bacterial tests. In the future, it could be a potential antibacterial agent against animal pathogenic bacteria.
Keywords: secondary metabolites, Actinomycetes, seaweed.

INTRODUCTION

The infection of bacterial diseases on a young animal will be resulting a compromised growth performance and high mortality. The effects of bacterial diseases infection on an animal are ranged from Listeriosis, Abortive Diseases, Mastitis, Polioencephalomacia, White Muscle Disease, and Pregnancy Toxemia (Yang *et al.,* 2015). The use of antibacterial chemicals continuously could cause a resistance to bacteria. The resistance of bacteria results in the ability to resist antibacterial by itself and still survive. Nowadays, antibacterial agents have been looking for bioactive compounds produced by seaweed for example.

Seaweeds or macro algae are kind of chlorophyll algae plants (Poncomulyo, 2006), enriched with vitamins and other compounds. Seaweeds have been used widely as a food, agar, alginate and carrageenan. Seaweeds also produce secondary metabolites which enable to self-defense against a predator, prevent ultraviolet radiation, as a competition tools and antibacterial (Harper *et al.*, 2001). The extract of *Turbinaria conoides, Padina gymnospora* and *Sargassum tenerrimum* have an antibacterial activity to attack *Enterococcus* sp., *Aeromonas* sp., *Staphylococcus aureus*, *Bacillus subtilis*, and *Salmonella* sp. (Manivannan *et al.*, 2011). In other hand, extract of *Padina* sp., *Sargassum* sp., *Gelidium* sp. could inhibit *Bacillus subtilis* activity (Marhaeni and Meinita, 2006). However, exploration of bioactive compounds of seaweed could lead to its biomass and over-exploitation, thus we need to find sources of natural antibacterial which environmental friendly and conservative.

Naturally, seaweeds can live together with microorganisms. These symbionts could produce the resemble or the same compounds produced by its host (Kelecom, 2002). Generally, the symbiosis of marine bacteria and marine organisms has a greater probability of producing chemical compounds than marine bacteria which live freely (Mearns-Spragg *et al.*, 1998). Utilization of bacterial symbiont with seaweeds as an antibacterial agent is an alternative to prevent over-exploitation and can be considered as the most conservative method (Nofiani *et al.*, 2009). The objective of this study was to evaluate the antibacterial

activities of Actinomycetes symbiont with seaweeds from Pangandaran beach, Indonesia against pathogenic bacteria for animal antibacterial.

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MATERIALS AND METHODS

Sample collection and isolation of Actinomycetes

Seaweeds *Halimeda macroloba, Gelidiella acerosa,* and *Turbinaria ornata* were collected from Pangandaran beach Indonesia. Fresh seaweeds were rinsed using sterile sea water to remove any associated epiphytes, salt, sand, microorganisms and other suspended materials. Then, 2.5 cm tissues of seaweed were cut and added to 5 mL of sterile seawater (serial dilution up to 10^{-3}). 0.1 ml of diluted sample was plated on Strach Nitrate Agar medium by pour plate technique and incubated at 30 ^{0}C for 7 - 10 days. On the basis of morphological features, colonies were randomly picked up and purified by using streak plates method (Pikoli *et al*., 2000).

DNA Isolation

Pure isolates of Actinomycetes were cultured in starch nitrate broth at 28 ^{o}C in a shaker (200-250 rpm) for 12 days, and the total genomic DNA of each strain was extracted using organic solvent (400 µl TE, 400 µl SET, 50 µl C TAB, 50 µl SDS, 167 µl NaCl, 400 µl cloroform, isopropanol, and 20-50 uL TE) (Nathwani, 2005).

Amplification of the 16S ribosomal DNA gene

16S ribosomal DNA gene amplification was performed using PCR primer BOX A1R (5'-CTACGGCAAGGCGACGCTGACGCTGACG-3'), 27F (5'-AGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). The PCR programs were done, initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1,5 min, extension at 68°C for 8 min and the final extension at 68°C for 10 min.

Extraction of antimicrobial compounds

The selected isolates from the representative cluster dendogram were inoculated into starch nitrate broth, and incubated at 28 ^{o}C in a shaker (200-250 rpm) for 12 days. After incubation the broths were filtered through Whatman No.41 filter paper, then the filtrate was extracted using ethyl acetate in the ratio 1:1 (v/v) and shaken well for 10 minutes. The ethyl acetate phase was separated and evaporated. The residue was weighed and redissolved with few ethyl acetate.

Bioassay of antibacterial activity

Six isolates were selected for its bioassay antibacterial activity, using *E. coli, Proteus* sp., and *Enterobacter* sp. from microbiology laboratory, Diponegoro University, Semarang, Central Java, Indonesia as the microorganisms test. Antibacterial activity was assayed in duplicate using standard paper disc method (Bauer *et al*., 1996). The dried crude extracts were dissolved in 100 ul etyle acetate. The samples (15 ul) were placed onto paper disks (6 mm) then onto the agar surface that contained the microorganisms test and incubated at 37 ^{0}C for 24 h. The diameters of any inhibition zone forms around the paper disks were measured using a caliper.

RESULTS AND DISCUSSION

Isolation of bacteria and genetic relationship

A total of 41 isolates of Actinomycetes were isolated from *Halimeda macroloba* (RL4), *Gelidiella acerosa* (RL5), and *Turbinaria ornata* (RL6) (Figure 1); 4 isolates from RL 4, 9 isolates from RL 5 and 30 isolates from RL 6. Actinomycetes were isolated using Stach Nitrate Agar (SNA). This medium seems to be specific and sensitive for Actinomycetes since it contains starch that most Actinomycetes use as a carbon source (Atta *et al.,* 2009). The highest diversity of Actinomycetes were found from *T. Ornata* (RL 6), it shows that *T. ornata* would be a host for Actinomycetes that live symbiotically in an amount more than the others. The community of bacterial symbiont on *Turbinaria* sp. tissue is more abundant than *Galaxea* sp. (Lee 2006).

The value of DNA purity of all isolates ranged from 1.2 to 3.6. The purity value of 1.8 to 2.0 indicates high purity, while the purity value below 1.8 is considered low and it is caused by contamination of high molecular such proteins. DNA purity value above 2.0 is considered high, it is caused by contamination of low molecular such RNA (Ausubel *et al.,* 1995; Sambrook *et al.,* 1989).

Genetic analysis showed that Actinomycetes had a band length between 100 – 3000 bp (Figure 2). The location of the same band has a close genetic relationship, it would be ease to distinguished of the relationship between isolates directly. Rep-PCR method could be used to identify the genetic relationship between sample by band position (Lavanya and Veerappan, 2001). Dendogram tree showed that all isolates created 3 clusters (Figure 3). Six isolates were chosen from 3 clusters (it was chosen as representative for all clusters). The isolates code are RL4 3, RL4 9, RL5 50, RL5 66, RL6 193, and RL6 206.

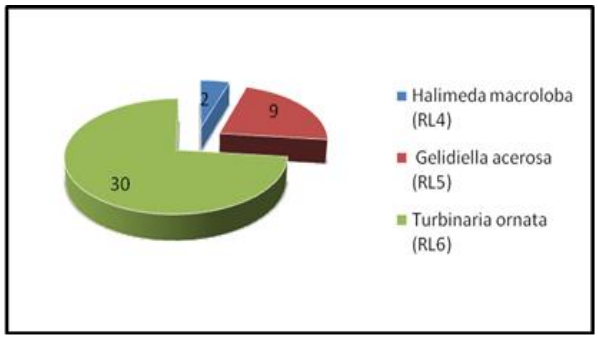

Figure 1. Total Isolates of Actinomycetes symbiont with *Halimeda macroloba* (RL4), *Gelidiella acerosa* (RL5), and *Turbinaria ornata* (RL6)

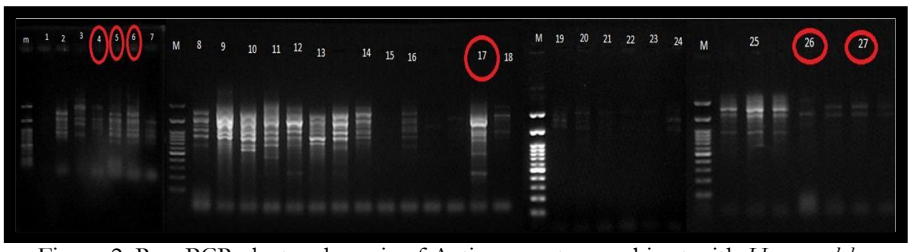

Figure 2. Rep-PCR electrophoresis of Actinomycetes symbiont with *H. macroloba, G. acerosa,* and *T. ornata.*

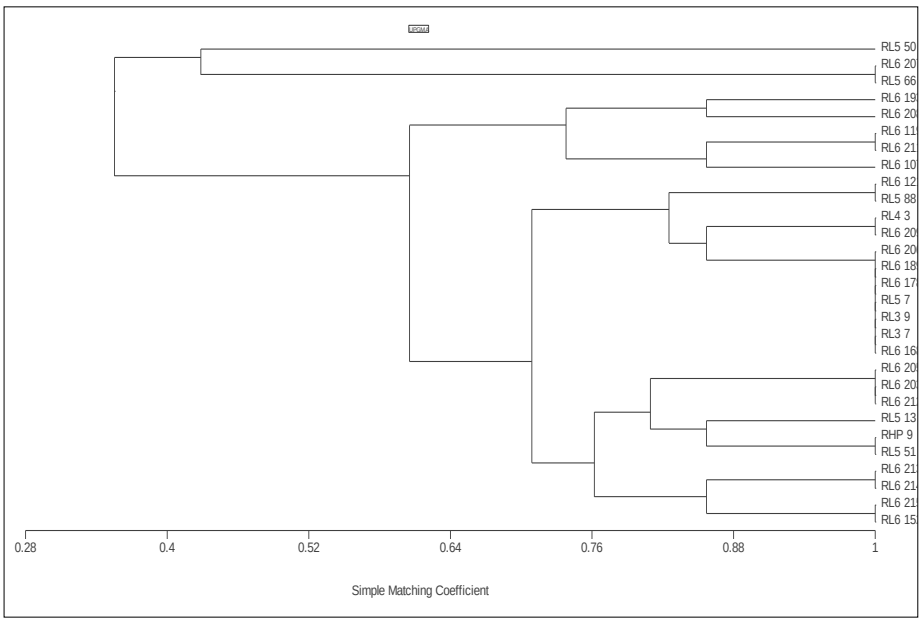

Figure 3. Dendrogram tree of Actinomycetes symbiont with *H. macroloba* (RL4), *G. acerosa* (RL5), and *T. ornata* (RL6).

The samples were selected based on the similarity coefficient <1, it may indicate far genetic relationship. The value of similarity coefficient 1 showed close genetic relationship and similarity coefficient <1 indicate a far genetic relationship. Rep-PCR is widely used to differentiate the isolates up to the level of species, subspecies and strain quickly and specific, also it can be used for grouping of bacteria (rapid grouping) (Rademaker and De Bruijn, 1997).

Antibacterial activity

Inhibition zone of RL4 3 against *Proteus* sp. showed the largest zone (9,12 mm), and RL4 9 had largest inhibition zone against *Enterobacter* sp. (6,45 mm) (Tabel 1). Isolate RL4 3 had more potential as antibacterial than RL4 9, it was shown by inhibition zone on all bacterial test. The extract of *Halimeda* sp. had a potential as antibacterial against *Proteus* sp. with inhibition zone 5,5 mm (Karthikaidevi *et al.,* 2009). It indicates that *H. macroloba* within its Actinomycetes symbiont had the same secondary metabolites against *Proteus* sp.

The result of inhibition zone of all Actinomycetes symbiont with *G. acerosa* (RL5 50 and RL5 56) had a great result against *Enterobacter* sp. It showed that all isolates had a potential as antibacterial agent against *Enterobacter* sp. Nowadays, the extract of *G. acerosa* have been used as antibacterial against *B. cereus, M. flavus, K. pneumoniae*, *P. testosteroni, C. Freundii* (Nair *et al*., 2007). Furthermore, Marhaeni and Meinita (2006) reported that *G. acerosa* have a potential as antibacterial against *B. cereus.* All isolates which are symbiont with *T. ornata* (RL6 193 and RL6 206) showed the best result against *E. coli.* The extract of *Halimeda* sp. has a potential as antibacterial against *E. coli* (Kolanjinathan and Stella, 2009). It indicates both *T. ornata* and Actinomycetes symbiont had the same secondary metabolites against *E. coli.*

Table 1. Inhibition zone from secondary metabolites of Actinomycetes symbiont with *H. macroloba* (RL4), *G. acerosa* (RL5), and *T. ornata* (RL6)

Isolates	Inhibition zone (mm)		
	E. coli	*Proteus* sp.	*Enterobacter* sp.
RL4 3	8.11 ± 2	9.12 ± 2	8.96 ± 2
RL4 9	5.56 ± 0.71	5.95 ± 2.64	6.45 ± 1.55
RL5 50	6.47 ± 1.20	6.72 ± 0.81	7.86 ± 2.79
RL5 66	5.16 ± 2	5.56 ± 2	5.89 ± 1
RL6 193	7.34 ± 5.33	6.96 ± 2.45	7.03 ± 1.52
RL6 206	7 ± 0.32	6.89 ± 2.07	6.91 ± 2
Range	5.16 – 8.11	5.56 – 9.12	5.89 – 8.96
Average	6.6	6.87	7.18

Maximum inhibition capabilities of each isolate against three bacterial tests can be informed that all isolates of Actinomycetes had potential as an antibacterial. Actinomycetes symbiont with *H. macroloba* had a higher potential against *Proteus* sp. and *Enterobacter* sp. The Actinomycetes isolates symbiont with *G. acerosa* had higher potential against *Enterobacter* sp. and Actinomycetes symbiont with *T. ornata* had higher potential against *E. coli.* The most potential ability of all isolates as a producer of antibacterial was RL4 3 which is symbiont with *H. macroloba.* This was proven by the amount of the maximum inhibition zone on all three bacterial tests. In other hand, *H. macroloba* had the fewest Actinomycetes symbiont. It showed that the seaweeds which have more isolates Actinomycetes did not show a high inhibition zone, but *H. macroloba,* although its only had two isolates Actinomycetes which is isolated, shown maximum inhibitory potential.

CONCLUSIONS

41 Actinomycetes were isolated from three seaweeds; 4 isolates from *Halimeda macroloba*, 9 isolates from *Gelidiella acerosa,* and 30 isolates from *Turbinaria ornata.* All isolates had antibacterial activities against *E. coli, Proteus* sp., and *Enterobacter* sp. Actinomycetes symbiont with *Halimeda macroloba* presented the largest inhibition zone on 3 bacterial tests.

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