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Diversity and Antifungal Activity of Actinomycetes Symbiont Hard Coral Mucus of Genera Goniopora and Porites

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Abstract

Screening new bioactive compounds from marine actinomycete organisms associated with corals (Goniopora and Porites) can be an alternative method to discover the natural antifungal compounds. This study aims to determine the density and diversity of actinomycete symbionts based on repetitive sequence-based-polymerase chain reactions (*rep*-PCR) and to discern the ability of antifungal activity of isolates symbiotic with hard coral mucus by using a pour plate method. A total of 143 isolates were obtained from the hard coral mucus of genera Goniopora and Porites. High genetic diversity was observed among the isolates. Ten isolates with different morphological characteristics were selected to extract its secondary metabolites and then followed by an antifungal test. The isolate with the code of SCAS324 was the one with the antifungal activity, marked by the formation of a very strong inhibition zone of 54.7±0.4 mm toward *Aspergillus flavus* and 49.2±2.7 mm toward *Candida albicans*. Antifungal screening showed that the antifungal activity of the isolate SCAS324 was three times as effective as the commercial antifungal.

Abstrak

Keragaman Aktivitas Antijamur Aktinomisetes Simbion Mukus Karang Keras Genus Goniopora dan Porites. Proses penapisan senyawa bioaktif baru dari aktinomisetes laut yang berasosiasi dengan organisme karang (Goniopora dan Porites) dapat menjadi metode alternatif untuk menemukan senyawa anti jamur alami. Penelitian ini bertujuan untuk mengetahui densitas dan keragaman simbion aktinomisetes berdasarkan *repetitive sequence-based-polymerase chain reaction* (*rep*-PCR) dan mengetahui kemampuan aktivitas anti jamur isolat yang bersimbiosis dengan mukus karang keras dengan menggunakan metode *pour plate.* Sebanyak 143 isolat aktinomisetes diperoleh dari mukus karang keras genus Goniopora dan Porites. Hasil rep PCR menunjukkan tingginya keragaman isolat. Sepuluh isolat dengan karakteristik morfologi yang berbeda dipilih untuk diekstraksi senyawa metabolit sekundernya yang kemudian dilanjutkan dengan uji anti jamur. Isolat dengan kode SCAS324 merupakan isolat yang memiliki aktifitas anti jamur, yang ditandai dengan pembentukan zona hambat yang sangat kuat sebesar 54,7±0,4 mm terhadap *Aspergilus flavus* dan sebesar 49,2±2,7 mm terhadap *Candida albicans.* Hasil penapisan anti jamur menunjukkan bahwa aktifitas anti jamur isolat SCAS324 tiga kali lebih efektif dibanding antijamur komersial.

Keywords: actinomycetes, antifungal, hard coral, mucus, rep-pcr

Introduction

Secondary metabolites in coral, that is, Goniopora and Porites, are formed as a response to environmental conditions. For instance, antimicrobial compounds are developed as a response to the invasion of fungi and bacteria that cause aspergillosis disease in coral [1]. In the process, the antimicrobial compounds are also produced by microbes associated in coral surface mucus [2]. Microbes that live in coral mucus are known to play the important roles in maintaining life and in encountering ecosystem pressures by deterring the [3] invasion of pathogens and the threat of predation and diseases through the production of antimicrobial compounds [2].

One of the microbial symbionts of coral mucus is actinomycetes [4]. Actinomycetes are Gram-positive bacteria known as the source of more than 10.000 antibiotic compounds [5]. For example, one study suggested that actinomycetes that are symbiotic with coral mucus from *Acropora digitifera* and that have antimicrobial activity can block the growth of pathogenic

bacteria, including *Staphylococcus aureus, Pseudomonas aeruginosa, Aeromonas hydrophila, Vibrio parahaemolyticus,* and *V. vulnificus* [4]. Nevertheless, the study of marine actinomycetes isolated as symbionts of hard coral mucus with potential as antifungal agents is very limited. Considering the high diversity of coral in Indonesia, with all the forms of association therein, screening of new bioactive compounds from marine actinomycetes associated with coral organisms (Goniopora and Porites) can be an alternative method for discovering antifungal compounds to overcome the problems caused by human pathogenic microbes, especially fungi or mildew. The discovery will be beneficial, since the increasing emergence of antifungal compounds is a serious challenge to modern medicine all over the globe. Moreover, nowadays, there is urgency to find alternative antifungal agents by using bioprospecting approaches.

Methods

Sample collection. The sample of this research was collected by employing a purposive sampling method, which means that a mucus sample was taken *in situ* from two different kinds of hard coral species in a healthy condition [4]. Mucus samples were collected from three different corals by using 10 mL of sterilized injection. Then, they were stored in sterile sample plastic containers [6] and transported to the laboratory at a temperature of ±4 °C.

Coral identification. Coral species were identified through visual observation that took into account morphology and other characteristics [7-8].

Isolation of Actinomycetes. A series of dilution methods were used for the isolation and purification of actinomycetes [9]. Five mL of mucus was placed in a tube that contained 5 mL of sterile sea water and then homogenized [6]. The sample was transferred into a test tube containing 4.5 mL of sterile sea water at 10^{-1} dilution and continuing to 10^{-4} dilution. Furthermore, 0.5 mL of each dilution was spread out on two media, starch nitrate agar (SNA) and starch casein agar (SCA), with pH between 7.2 and 7.4 [9]. The samples were incubated at a temperature of 30 °C for four weeks. The grown colonies of actinomycetes were separated and transferred into a new medium to obtain single colonies. A Gram rapid test was conducted briefly by taking 1 dose of pure actinomycetes isolate and then mixing it with 2 drops of 3% KOH. Next, it was stirred continously by using a needle to form a suspension loop. Sticky mucus appeared when the suspension was lifted by using a loopful of Gram negative (-) bacteria [10].

Isolation of DNA. DNA was isolated by using phenol-chloroform. The previously cultured actinomycetes in 1 mL of liquid were taken and centrifuged (IEC Micro-EB Centrifuge USA) at 13.000 rpm for five minutes. Pellets were added to a solution of *Tris-HCl* TE with 400 mL ethylenediaminetetraacetic acid (EDTA) and centrifuged for five minutes. Furthermore, 50 mL of 10% sodium dodecyl sulphate (SDS) was added and incubated at 65 °C. After that, 167 mL of NaCl solution was added and incubated at 65 °C for an hour. Next, 400 mL of cold chloroform solution was added and incubated for 30 minutes. Then, the sample was centrifuged at 13.000 rpm room temperature for 10 minutes, and a solution of isopropanol was added. The sample was centrifuged for five minutes, and the pellet was homogenized with 100 mL of 70% ethanol. Ethanol was evaporated until the pellets were dry. The pellets (DNA) were resuspended in 20 mL of TE (Tris –EDTA) solution [11-12].

Repetitive extragenic palindromic-PCR (rep-PCR). Repetitive DNA fingerprinting was performed for isolates based on the method of Sadowsky *et al*. (1996). Primary BOXA1R PCR derived from the repetitive sequences (5'-CTACGGCAAGGCGACGCTGACG -3') was used to amplify the DNA samples. The PCR mixture contained 3 µL of H_2O, 1 µL of DNA genome of actinomycetes as a template (50 ng/µL), 1 µL of primary BOX A1R (15 pmol), and 5 µL of Mega Mix Royal (MMR). The following PCR condition was performed: pre-denaturing (95 °C for 5 minutes), 30 cycles of 94 °C for a minute, 53 °C for 1.5 minutes, 68 °C for one minute, and a final extension step at 68 °C for 10 minutes. Amplification products were analyzed by 1% electrophoresis (agarose gels were stained with ethydium bromide).

Extraction of secondary metabolites. Actinomycetes were cultured in 500 mL of a liquid SNA medium for 12 days [13]. The liquid culture was centrifuged at 150 rpm for 20 minutes. Ethyl acetate was added to the supernatant to obtain a volume ratio of 1 : 1, and then it was extracted and evaporated.

Antifungal activity test. Antifungal activity testing was conducted by employing a modification of paper disc method [14]. *Aspergillus flaveus* and *Candida albicans* were used for the fungi test. A total of 10 µL of ethyl acetate, extract (2000 µg/mL), and commercial antifungal were dropped into a 6-mm-diameter paper disc. Incubation was carried out at 30 °C for 24 hours. Antifungal activity was shown by a clear zone around the paper disc [9].

Results and Discussion

Coral identification showed three types of coral samples originated from genera Goniopora and Porites of the Poritidae family. The Poritidae family is a group of corals that live in colonies and have massive sizes with various shapes. The genus Goniopora includes free-living colonies that are massive and flat shaped and that are encrusted with relatively large and thick corallite. In our study, septa and columella united to form a compact structure. The colony had a long polyp shape, and the

corallite was small and of a cereoid type. Septa united with each other and formed a very typical structure (known as a pali). It showed different colors, such as brown, yellow, brown with white, light green, and grey. The distribution varied throughout Indonesia in the shallow waters or reef areas [8].

A total of 79 isolates were derived from the Goniopora genus, and 64 isolates were from the Porites genus. The number of actinomycetes of symbiont hard coral mucus from the genus Goniopora was higher compared to that from the genus Porites (Table 1). The high diversity and abundance of coral mucus isolates showed that the media used were SNA and SCA. Both of them contained sources of carbohydrates and nitrogen that were required for the growth of actinomycetes. In addition, sea water was used as a solvent to provide the requirement for complex ions that made the media very suitable for the growth of marine microbes, especially actinomycetes [16].

The number of bacteria isolated from the mucus of coral *Fungia scutaria* was 43, and 11% of them were from actinomycetes. For *Platygira lamellina*, 51% of 36 isolates were obtained from actinomycetes [15]. The percentage of actinomycetes from the obtained 22 isolates was about 23% [17]. For the coral *Acropora,* 24 isolates were isolated from mucus; however, only about 4% of them belong to actinomycetes [18]. For the coral *Acropora cervicornis*, about 30 bacterial isolates were isolated from mucus. They consisted of four main groups, namely, *gamma proteobacteria*, *alfa proteobacteria*, *cyanobacteria*, and *actinobacteria* [19]. No reference was found about actinmycetes symbionts of the Goniopora and Porites genera.

Ten isolates were selected based on their low similarity coefficient value (<< 1) (Figure 1). Based on this dendogram, high genetic diversity was observed among the isolates. A similarity coefficient value that was less than 1 indicated a distant genetic relationship, while a similarity coefficient of 1 showed a closer one [20].

After the 10 selected isolates for fungi test (*A. flavus*) were observed, the average of the inhibitor zone diameters of their secondary metabolites showed that isolate SCAS324 (Figure 2a) could inhibit *A. flavus* fungi. The average of the inhibition zone diameter was classified as high (about 54.7 mm), while the extract concentration was 2000 mg/mL (Figure 2b). The inhibitory zone of K1, the first control that used a commercial antifungal, was classified as the moderate category (only 11.7 mm). The second control, K2, which was the ethyl acetate solvent, showed no inhibitory zone at all. The absence of an inhibition zone in K2 showed that the antifungal activity came from the compound produced by isolate SCAS324 and was not originated from the solvent (Table 2). In addition, this compound possessed the better ability to inhibit the fungi in tests than the commercial antifungal compound, which means its use could be reconsidered as the source of a natural antifungal that could replace the synthetic ones.

Table 1. The Number of Isolates that are Symbiont with Hard Coral Mucus from Genus Goniopora (S1 and S2) and Genus Porites (S3)

Sources of Isolates in Hard Coral	Number of Isolates in Medium		Total
	SNA	SCA	
Genus *Goniopora*	10	69	79
Genus *Porites*	44	20	64

Table 2. Mean Inhibition Zone Diameter (antifungals) of Coarse Ethyl Acetate Extracts of 10 Selected Isolates (K1: Antifungal Commercial, K2: Ethyl Acetate)

Isolate Code	Inhibitor Zone Diameter (mm)			
	K1	K2	*A. flaveus*	*C. albicans*
SNAS3241	10	0	0	0
SNAS3219	10	0	0	0
SNAS3211	10	0	0	0
SNAS329	8	0	0	0
SNAS122	8	0	0	0
SNAS3238	8	0	0	0
SCAS324	11.7	0	54.7 ± 0.4	49.2 ± 2.7
SCAS113	11.7	0	0	0
SCAS2161	11.7	0	0	0
SCAS137	11.7	0	0	0

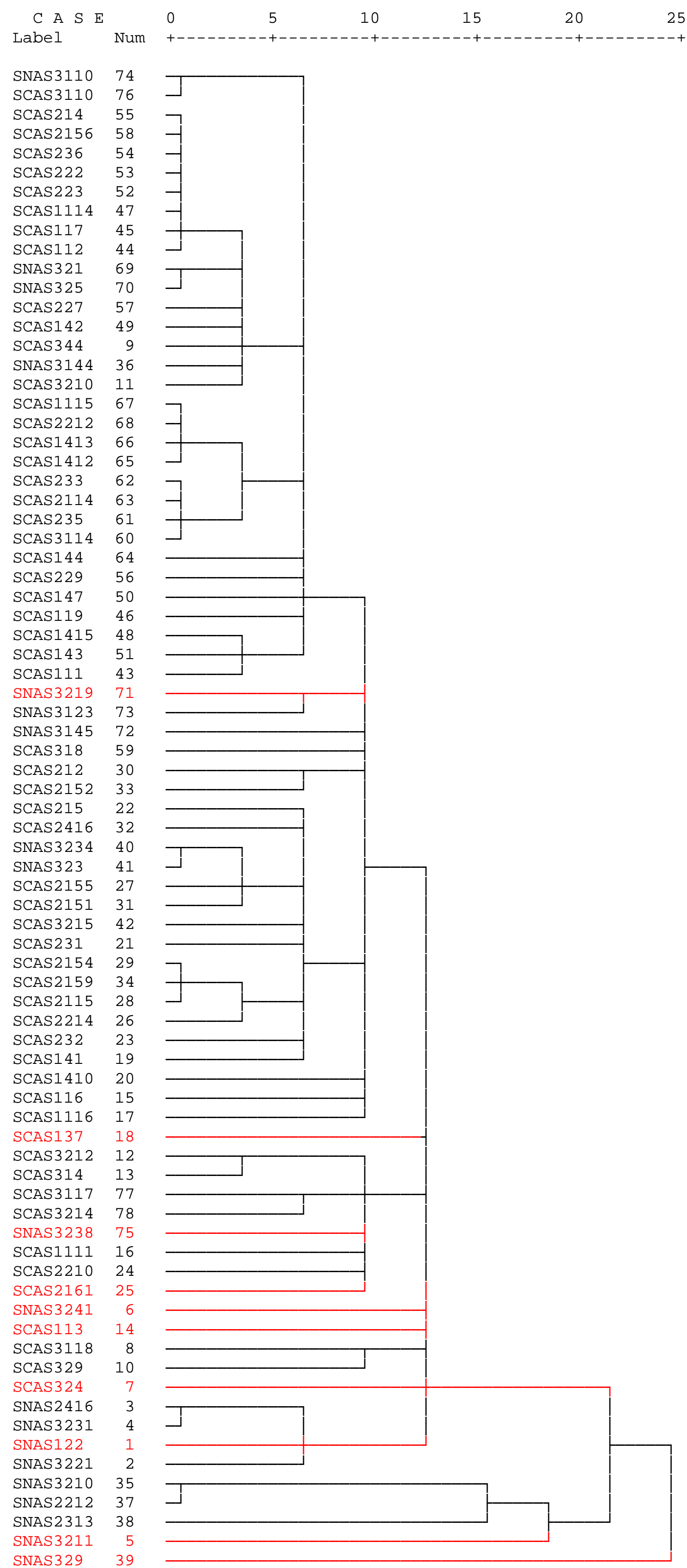
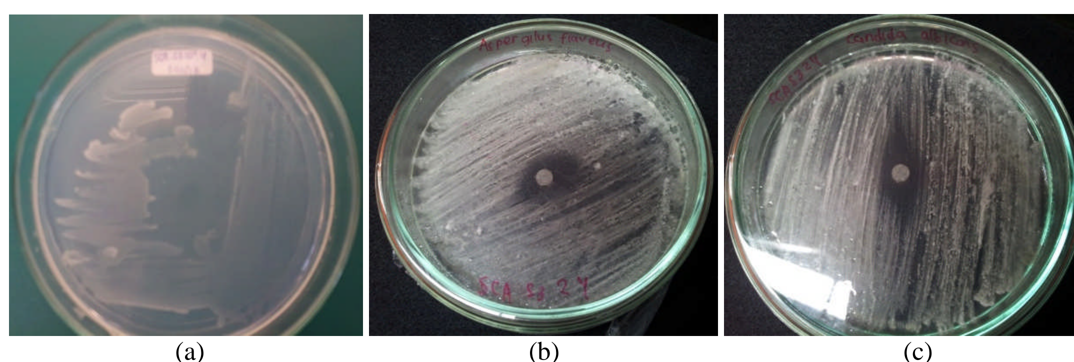


Figure 1. Results of Groupings of Dendogram Actinomycetes Isolates; the Isolates Code is Printed in Red, Representing the Antifungal Test



(a) (b) (c)

Figure 2. (a) Isolate SCAS324, (b) Zone of Inhibition Against *A. flavus* (mm), (c), and Zone of Inhibition Against *C. Albicans* (mm)

Actinomycetes isolates from *Streptomyces albogriseolus* and *S. xiamenensis* that were isolated from the coral *Anthipates dichotoma* are known to have high antifungal activity toward *A. versicolor* and *A. sydowii* in the range of the inhibition zone of 7.6 to 14.3 mm [21]. Ali (2009) was successful in isolating 47 actinomycetes isolates of sago. Approximately 30 isolates carried the antifungal activity (primarily from the genus Streptomyces) [9]. *Streptomyces* sp. showed large antifungal activity toward fungi *Aspergillus flavus.* The 11 mm inhibition zones were formed at the extract concentration of 10 mg/mL and 19 mm at the extract concentration of 30 mg/mL [22].

In addition to *A. flavus,* isolate SCAS324 was able to inhibit a second fungus in a test for *C. albicans,* with the same extract concentration, 2000 mg/mL. The average formation inhibition zone was relatively high, which was 49.2 mm (Figure 2c), while the size of inhibition zones of K1 and K2 were the same as the previous ones, 11.7 mm and 0 mm. The result showed that isolate SCAS324 contains a natural antifungal compound that is potentially valuable since it could inhibit fungi in both tests in the high inhibitory zone category. It was higher than the commercial antifungal available in the market.

Actinomycetes from *Streptomyces kanamyceticus, S. verse*, *S. narbonensis*, *S. malachitofuscus*, and *S. hygroscorpicus* carried the most potential inhibition property toward *C. albicans* [22]. Of 64 Actinomycete isolates, 12 (18.8%) possessed the ability to act as an antibacterial, 13 (20.3%) showed a propensity for antifungal ability toward fungal type *C. Albicans,* and 9 (14.1%) showed antibacterial and antifungal characteristics [23]. Actinomycetes that were isolated from mangrove sediments also showed antifungal activity toward *C. albicans* [24]. Sunaryanto *et al.* (2009) isolated actinomycetes from marine sediments collected from three different beaches. In their study, five isolates carried the ability to resist C. *albicans* fungi. The inhibition zone of extract SCAS324 was higher than the commercial antifungal used as the first control (K1), while commercial antifungal products gave a higher antifungal activity than the extract compound produced by Streptomyces [25]. This indicates that isolate SCAS324 could be a potential isolate in producing antifungal compounds.

Conclusions

A total of 143 pure isolates of actinomycetes were symbiotic with hard coral mucus; 79 of them were derived from the genus Goniopora, and the rest 64 were from the genus Porites. Screening of secondary metabolites showed a high antifungal potential for isolate SCAS324, as the high inhibition zones toward *A. flavus* and *C. albicans* were three times as effective as the synthetic antifungal.

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