Symbiotic Fungus of Marine Sponge Axinella sp. Producing Antibacterial Agent

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Abstract. The emerging of multidrug resistance pathogenic bacteria cause the treatment of the diseases have become ineffective. There for, invention of a new drug with novel mode of action is an essential for curing the disease caused by an MDR pathogen. Marine fungi is prolific source of bioactive compound that has not been well explored. This study aim to obtain the marine sponges-associated fungus that producing anti-MDR bacteria substaces. We collected the sponge from Riung water, NTT, Indonesia. The fungus was isolated with affixed method, followed with purification with streak method. The overlay and disk diffusion agar methods were applied for bioactivity test for the isolate and the extract, respectively. Molecular analysis was employed for identification of the isolate. The sponge was identified based on morphological and spicular analysis. The ovelay test showed that the isolate KN15-3 active against the MDR *Staphylococcus aureus* and *Eschericia coli*. The extract of the cultured KN15-3 was also inhibited the *S. aureus* and *E. coli* with inhibition zone 2.95 mm and 4.13 mm, respectively. Based on the molecular analysis, the fungus was identified as *Aspergillus sydowii*. While the sponge was identified as *Axinella* sp.

Keywords: Fungi, sponge, *Aspergillus sy*dowii, multidrug resistace, *Axinella* sp.

1. Introduction

Marine sponge is a source of bioactive compounds the pharmaceutical properties such as antioxidants [1], antibacterial [2], antifungal [3], anti-inflammatory [4,5], anti-malarial [6], and anticancer [7].

However, only few of them passed to the preclinical and clinical stages [8]. Compounds supply is a major problem for drug development from marine source [9]. One of the most serious bottlenecks in developing natural products from coral reefs has been the availability of biomass to gain sufficient amounts of substances for preclinical and clinical studies. Exploitation is further complicated by the fact that most of these metabolites possess highly complex structures, making them difficult to be produced economically via chemical synthesis. Sea culture is one alternative that may be used to solve the need of bioactive compounds from sponge though the sponge grows slowly [10,11]. Burgess *et al.* [12],

mentioned that bacteria symbiotically associated with soft corals can synthesize secondary metabolites similiar to host.

Sponge is a filter feeder animals so that microorganisms accumulate in large quantities as mesophyll. Some researchers believe that microorganisms play an important role, such as providing food, bioactive compounds, or precursors to their host [13]. Association number of bacteria in the sponge more than fungal symbionts, but fungal symbionts produce clinically active compounds is more important than bacteria [14].

This paper describes the isolation and identification of the associate fungus of marine sponge collected from Riung Water, East Nusa Tenggara, Indonesia.

2. Materials and methods

2.1. Sample collection

Marine sponges were collected from the Tujuh Belas Pulau, Riung, East Nusa Tenggara, Indonesia by SCUBA diving at 3-15 m depth. The specimens were kept in a cool box until inoculation process [15].

2.2. Isolation of fungi

Isolation of fungi on a sponge conducted with modification methods of Subramani *et al*. [16]. The sponges were washed with sterilized sea water prior to inoculation process to removed loosed associated microorganisms from its surface. Then, the specimen was cut into small piece (1 x 1 x 1 cm). The specimen laid on the surface of malt agar medium. After 7 days incubation, the fungus colonies were separated based on their morphological characteristics, and each colony was inoculated on a new agar plate contain MEA.

2.3. Antibacterial screening

Isolates of MDR bacteria, *Staphylococcus aureus* and *Escherichia coli*, was obtained from the Laboratory of Microbiology, Kariadi Hospital, Semarang. Culture of each MDR bacterium in the logarithmic phase was mixed with Zobell soft agar medium (1% v/v), which were then poured on to the respective agar surface previously inoculated with fungi symbionts sponge and incubated for 24 h [17]. The inhition zone indicated that isolates were active against the antagonistic bacteria (the data were not shown in this paper).

2.4. Sponge Identification

Identification sponge symbiont host of fungal isolates that produce antibacterial MDR *E. coli* and *S. aureus* based on morphological characteristics and the characteristics of spicules based methods of Hooper [18], with slight modifications. Morphological identification is done by color, surface shape, and the shape of growth.

Identification of the spicules is done by cutting a small fragment of the sponge and put in eppendorf microtub, then sodium hypochlorite was added. After the organic components were dissolved and leaving only the mineral skeleton, the bleach was diluted with ethanol and replaced carefully. The washing process was repeated several time. Spicules were taken and placed on a glass slide, and then observed under a microscope. the observations were documented by camera.

2.5. Extraction

The pure isolated shown active against *S. aureus* and *E. coli* were cultured on 1 L MEB media for 7 days or until the maximum growth at ambien temperature. The media was filtered to obtain the micellium that was extracted with methanol at room temperature. The solvent was filtered using filter paper and then dried with rotary evaporator [19].

2.6. Bioassay methanolic extracts

Bioassay methanolic extracts based on the method proposed by Safaeian *et al.* [20] with slight modification The extracts were tested againts the *S. aureus* and *E. coli* using difussion agar method at the concentrations 400, 200, 100, 50 and 25 µg/disk with two times repeated. The pathogenic bacteria were inoculated by spread method on MEA media, after 30 minutes incubation, the paper disks contained the methanolic extract were placed on the agar media surface.

2.7. Fungi KN-15-3 identification

2.7.1. Morphology identification

Fungi symbionts aged 7 days taken by ose needle and placed on a glass slide that has been etched with a solution of lactic acid then observed under a microscope with a magnification 40x. The results are documented using camera [21].

2.7.2. Molecular identification

The DNA isolate was extracted by *Wizard Genomic DNA purification Kit* following the instruction provided by the company (PROMEGA).

DNA engine thermal cycler (MyCycler, Biorad), PCR run comprised 34 cycle with cycle: initial denaturation at 95 ^{0}C for 5 minutes, annealing (57,1 ^{0}C for 1 min), extension (72 ^{0}C for 7 min), denaturation (94 ^{0}C for 40sec), and 42 ^{0}C for 1 min, 70 ^{0}C for 5 min, and the last 4 ^{0}C.

The DNA in supernatant was extracted with isopropanol for further analyses with electrophoresis on agrose gel. Electrophoresis was run at 100 volt with TAE (Tris-Acetate-EDTA) as running buffer. Ethadium bromida was utilized as pigment prior to visualization under UV light. The primers used for PCR amplification of 18S rRNA is universal primers ITS 1 (5'TCCGTAGGTGAACCTGCGG-3') and primer ITS 4 (5'TCCTCCGCTTATTGTATGC-3') [22]. Sequencing process was conducted in Macrogen, Korea.

3. Result and Discussion

From Riung Water of Nusa Tenggara Timur Province-Indonesia, a total of 18 sponges were collected that provided 33 fungus isolates. After antagonistic bioassay, the most active isolate, KN-15-3, was chosen for further study (the screening process was not performed in this paper).

The host of the isolate was also be identified base on morphological characteristics and spiculas analyses. Sponges host KN-15-3 has typical characteristicssuch such as in natural environment has a bright orange red color, while in alcohol brown or light brown, corrugated, with vertical or horizontal and branches in bush-shaped (Figure 1).

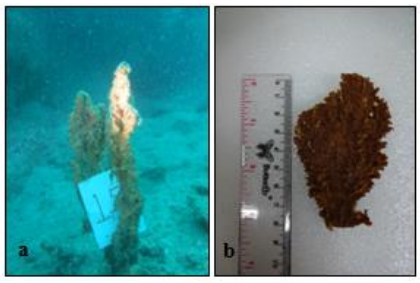


Figure 1. The photograph of the sponge K-N-15 (a). In situ (b). in the laboratory.

The identification of host sponge spicules KN-15-3 have type: style, subtylostyle, and strongyle (Figure 2). Based on the characteristics identification and spicules, sponges included in Genus Axinella [18, 23]. Genus Axinella known to be rich bioactive compounds such as anti-virus, cytotoxic, neurological activity, antioxidants and antifungals [24-28].

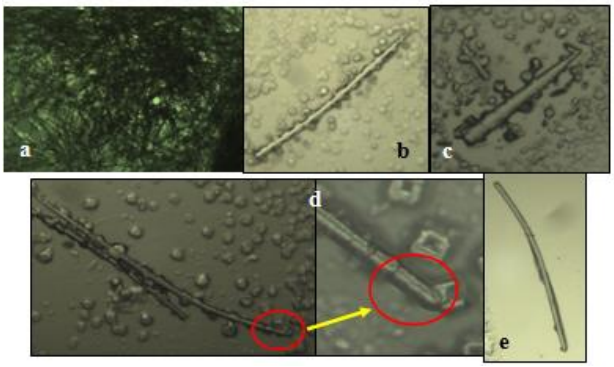


Figure 2. Spicules of the sponge K-N-15 (a = 10x, b-e = 40x) a= skeleton; b.,c. = Style; d = subtylostyle; e.= strongyle

On Malt extract agar media, fungal isolates have white mycelium and spores darker in color. On the observation by a microscope isolates KN-15-3, spores are round and surrounded by conidia thus forming like a flower (Figure 3).

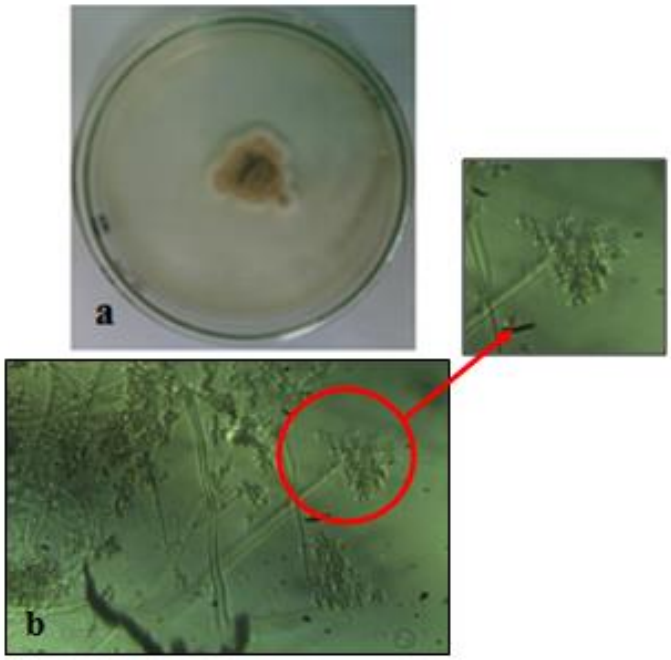
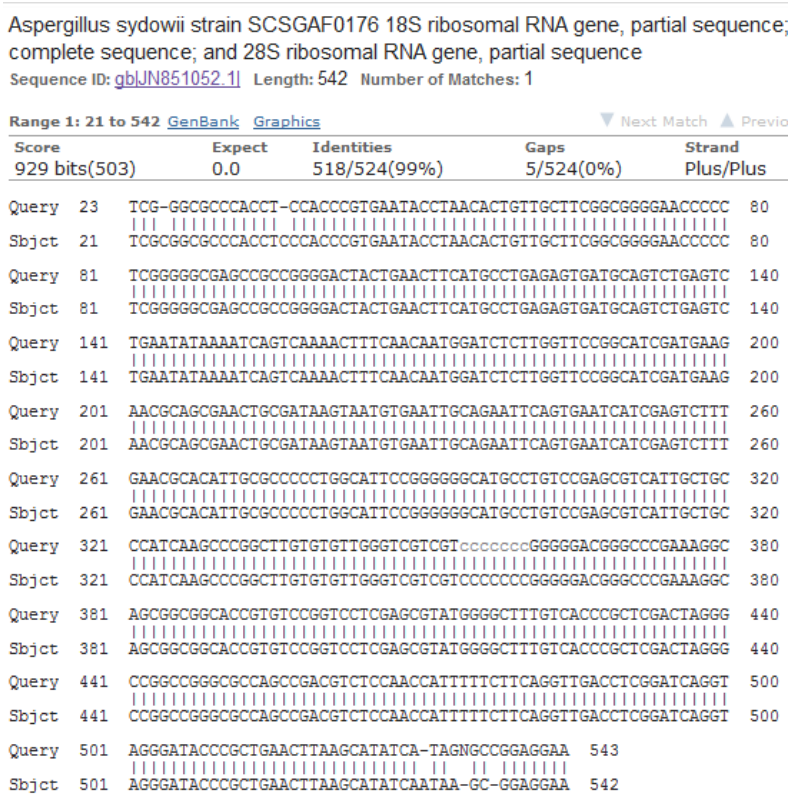


Figure 3. a. Fungi symbionts K-N-15-1. b. Mycelium of fungi symbiont sponge K-N-15-1

Based on the sequencing of fungal symbionts KN-15-3 has 99% similarity with the fungus Aspergillus sydowii (GenBank accession no. JN851052.1) (Figure 4). The isolate has been registered to GenBank with accession number LC094427.


Aspergillus sydowii strain SCSGAF0176 18S ribosomal RNA gene, partial sequence; complete sequence; and 28S ribosomal RNA gene, partial sequence

Sequence ID: gb|JN851052.1| Length: 542 Number of Matches: 1

Range 1: 21 to 542 GenBank Graphics Next Match Previo

Score	Expect	Identities	Gaps	Strand
929 bits(503)	0.0	518/524(99%)	5/524(0%)	Plus/Plus

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Query  23   TCG-GGCGCCCACCT-CCACCCGTGAATACCTAACACTGTTGCTTCGGCGGGGAACCCCC  80
            ||| |||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  21   TCGCGGCGCCCACCTCCCACCCGTGAATACCTAACACTGTTGCTTCGGCGGGGAACCCCC  80

Query  81   TCGGGGGCGAGCCGCCGGGGACTACTGAACTTCATGCCTGAGAGTGATGCAGTCTGAGTC  140
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  81   TCGGGGGCGAGCCGCCGGGGACTACTGAACTTCATGCCTGAGAGTGATGCAGTCTGAGTC  140

Query  141  TGAATATAAAATCAGTCAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAG  200
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  141  TGAATATAAAATCAGTCAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAG  200

Query  201  AACGCAGCGAACTGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTT  260
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  201  AACGCAGCGAACTGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTT  260

Query  261  GAACGCACATTGCGCCCCCTGGCATTCCGGGGGGGCATGCCTGTCCGAGCGTCATTGCTGC  320
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  261  GAACGCACATTGCGCCCCCTGGCATTCCGGGGGGGCATGCCTGTCCGAGCGTCATTGCTGC  320

Query  321  CCATCAAGCCCGGCTTGTGTGTTGGGTCGTCGTccccccGGGGGACGGGCCCGAAAGGC  380
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  321  CCATCAAGCCCGGCTTGTGTGTTGGGTCGTCGTCCCCCCCCGGGGGACGGGCCCGAAAGGC  380

Query  381  AGCGGCGGCACCGTGTCCGGTCCTCGAGCGTATGGGGCTTTGTCACCCGCTCGACTAGGG  440
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  381  AGCGGCGGCACCGTGTCCGGTCCTCGAGCGTATGGGGCTTTGTCACCCGCTCGACTAGGG  440

Query  441  CCGGCCGGGCGCCAGCCGACGTCTCCAACCATTTTTCTTCAGGTTGACCTCGGATCAGGT  500
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  441  CCGGCCGGGCGCCAGCCGACGTCTCCAACCATTTTTCTTCAGGTTGACCTCGGATCAGGT  500

Query  501  AGGGATACCCGCTGAACTTAAGCATATCA-TAGNGCCGGAGGAA  543
            ||||||||||||||||||||||||||||| ||  || |||||||
Sbjct  501  AGGGATACCCGCTGAACTTAAGCATATCAATAA-GC-GGAGGAA  542
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Figure 4. Results of sequence homology analysis isolates KN-15-3using the BLAST database.

The genus Aspergillus is the macroscopic filamentous fungi and one of the causes contaminants in food. This is caused by Aspergillus grows colonize and reproduce mycotoxins [29]. Aflatoxins are secondary metabolites produced by *A. flavus*, A. *parasiticus*, *A. nomius,* dan *A. tamarii* [29-32]. Aflatoxins can be found in many types of food and feed ingredients such as wheat, wheat flour, maize, cereals, rice, beans, spices, and beer [33-34].

The genus of Aspergillus is also known and recognized as a source of bioactive compounds in pharmacological field. Some bioactive compounds produced by Aspergillus genus such as antibacterial varixanthone, anticancer asperazine, antiparasitic gliotoxin 6-methoxyspirotryprostatin B, and antifungal Amphotericin B [35-38].

A.sydowii known to have some activity such as tyrosinase inhibitors, anthraquinone, as antimicrobial, sydowiols A and sydowiols C as anti *M. tuberculosis*, and as acetylcholinesterase inhibitor [39, 40]. Acetylcholinesterase inhibition resulted in increased concentrations of acetylcholine. This further result in increased communication between nerve cells that use acetylcholine as a chemical messenger producing a therapeutic effect in patients with Alzheimer's disease

In antagonistic test the isolate KN-15-3 showed active against two MDR bacteria *Staphylococus aures* and *Escherichia coli.* The methanolic extract of the isolate also exhibited strong activity against both pathogens with inhibition zone and 15.10 mm (*E. coli*) and 18.75 mm (*S. aureus*) at the concentration 400 μg/disk, so it can be developed as a source of new drug.

4. Conclusion

Isolates KN-15-3 produces antibiotic that inhibited the MDR *S. aureus* and *E. coli.* Based on the morphological analysis and molecular analysis the fungus isolate KN-15-3 identified as *Aspergillus*

sydowii. The morphological and spicular characteristics indicated that the sponge host, KN-15 was Axinella sp.

5. Acknowledgement

We thank Marine Diving Club members for their assistance during sponge collection. This Research was supported by The Ministry of Research, Technology, and Higher Education through Post Graduate Scheme Research Grant with contract number :139-01/UN7.5.1/PG/2015.

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