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EXPLORATION OF MARINE SPONGES-ASSOCIATED FUNGI PRODUCING ANTIFUNGAL COMPOUNDS

AGUS TRIANTO[1,2]*, AGUS SABDONO[1], BASKORO ROCHADDI[1] AND DESY WULAN TRININGSIH[2]

[1] Department Marine Science, Faculty of Fisheries and Marine Science, Diponegoro University, Semarang, Indonesia, 62-247474698
[2]Natural Product Laboratory, Centre for Research and Services (UPTLT), Diponegoro University, Semarang, Indonesia

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Abstract - The morbidity of diseases caused by fungi is still considered high in Indonesia due to the hot and humid climate that suitable for the fungi growth. The unwise treatment for the diseases using antimicrobial caused a new problem of the development of the resistant strain. A vast distribution of multi-drug resistant pathogens urged to discover new antifungal compounds. The purpose of this study is to obtain the marine sponge-associated fungi that produce antifungal substances. A total 67 sponges specimens were collected from four different areas in Indonesian water. The antagonistic test was conducted against *Malassezia furfur, Trichophyton* sp, and *Candida albicans* using cross streak method for screening the active isolates.The sponge-isolates ratio from Lampung Bay, Seribu Islands, Karimunjawa Islands and Wakatobi Island were 106%, 90%, 210%, and 115%, respectively. The sponge collected from Wakatobi Islands has the highest number of the active isolates against the *M. furfur*, *Trichophyton* sp, and *C. albicans*. The extract one of the isolated fungus showed activity against all of the pathogenic fungi at the concentration as low as 100 μg/disc.

INTRODUCTION

The prevalence of fungal infections in a tropical area, especially related to the skin diseases, are very high and infect almost all social levels. The infection becomes higher in the isolated tribes, rural and coastal areas where the sanitation and nutrition are bad. A study conducted in the West Java coastal area showed that dermatomycosis and dermatophytosis diseases are the main diseases infect the people in that area (Bramono *et al*., 2008). The diseases transmission is usually through skin contact, or from the clothes contaminated with fungal spores. In human skin, fungi live in the keratin layer of the epidermisand able to disengage toxin that causes inflammation and irritation. The genus *Malassezia, Trichophyton*, and *Candida* are well known as pathogenic and parasitic fungi in humans (Gupta *et al*., 2003; Gupta *et al.*, 2004; Tan, 2005). *Candida* sp. is a yeast which causes candidiasis that infects the mouth or vagina. If the yeastis entering the blood, they caused a disease which is potentially life threatening. *Trichophyton* sp. is a fungus causing skin diseasemarked by a ring-shapedblotch that can occur in arms, legs, neck, or body (Piérard *et al*., 1996).

In 2008, the new patients under illness cause group "Skin Disease and Subkutan Tissue" is one group of outpatients according to the International Classification of Diseases - 10 (ICD-10) (Departement of Health of the Republic of Indonesia, 2009). Furthermore, the Indonesia Health Profile 2010 showed the skin diseases and subcutaneous tissue become the third out of 10 most occuring diseases of outpatients that indicated the skin diseases has been growing (Ministry of Health of the Republic of Indonesia, 2011). Most of the treatments will remove the active infection evidence (squama) in a few days, but to assure a thorough treatment this strict treatment have to be

***Corresponding author's email-** agustrianto.undip@gmail.com

continued for a few weeks. However, a long-term treatment can cause damage to the tissue, particularly in the liver (Healy and Barnes, 2006). Besides, the eficacy from those medicines also decreasing along with the emerging of the resistance from pathogen fungi.

The coral reef ecosystem is a bioactive resource, including antifungal compounds which have not been optimally used yet. A variety of marine invertebrates such as sponges, soft corals, and ascidians are proven to produce bioactive material; however, the utilization is constrained by the material supply. Sponge Hyrtos sp. produces alkaloid hyrtimomines D and E compounds which are able to block *Candida albicans* (IC50, 4 and 8μg/mL, respectively), while the hyrtimomine D is able to block *Trichophyton mentagrophytes* (MIC, 16 μg/mL) (Tanaka *et al*., 2013). However, to produce 1 mg of the bioactive compound, 1 ton marine invertebrates are needed. In the prior study, we isolate fungi from sponges that could produce antifungal compound with a stronger activity than flukanol, antifungal medicine available in the market. In addition, a more extensive and in-depth exploration is needed to look for a new antifungal medicine.

METHOD

The Collection of the Sponges Specimens

The sponges were collected using SCUBA diving method at a depth of 3 to 40 m in Lampung Bay, Seribu Islands, Karimunjawa Islands and Wakatobi waters. The collected specimen were kept in sterile plastic bags until isolation of the fungi (Trianto *et al*., 2011a; Trianto *et al*., 2011b; Trianto *et al*., 2014).

Isolation of Associated Fungi

Fungi associated with marine organisms were inoculated in media by putting a part of the sponges in MEA (Malt Extract Agar) aseptically. Those media then were incubated at room temperature until the fungi grew. Then the growing fungi are inoculated in a slant media prior to the antagonistic test.

Isolation and Purification of Fungi

Isolation of the fungi were conducted by isolating the colony of fungi aseptically to be moved to new media. This isolation process is conducted several times, so the produced isolates were strongly pure.

Antagonistic Test

The antagonistic test is used to screen the active isolates that are indicated by inhibition zone of the pathogenic fungi, i.e. *Malazzesia furfur, Trichophyton* sp, and *Candida albicans* obtained from Diponegoro National Hospital. The antagonistic tests were conducted using cross streak (Mohanraj *et al*., 2011). In this method the isolated streaked perpendicular to the three pathogenic fungi. Some isolates were also tested by using agar overlay (Fleming *et al*., 1985) or agar plug method (Monthon, 2005).

Phenotypic Characterization

The potential isolate was grown on MEA media to examine and photograph the morphological characteristics under a microscope. Isolate identification was conducted using a fungal taxonomy book and taxonomic guidance (Seifert *et al*., 2010).

RESULTS

Sponge Collection and Isolation of Marine Fungal Symbiont

This study report marine-derived fungi that associate with sponges. A total of 84 marine fungi were isolated from 67 sponges obtained from four different locations: Lampung Bay, Seribu Island, Karimunjawa Islands, and Wakatobi Islands (Figure 1).

The sponges from Karimunjawa Islands hosting the highest fungi compare to the other sponges. The number of the isolated fungi was double from the number of sponges. This result may correlate with the difference of fungal populations that exist within different sponge species and the difference of the geographic locations. The number of fungal isolates and its host in this study is shown in Figure 2.

Screening for Antifungal Activity

Primary Screening Test

Searching on antifungi from marine fungi was started by conducting antifungal test against *Candida albicans*, *Trichophyton* sp., and *Malassezia furfur*. The antagonistic test was carried out by using cross-streak (Table 1), agar plug, or agar overlay method (data is not shown). The number of the active isolates is shown in Figure 3.

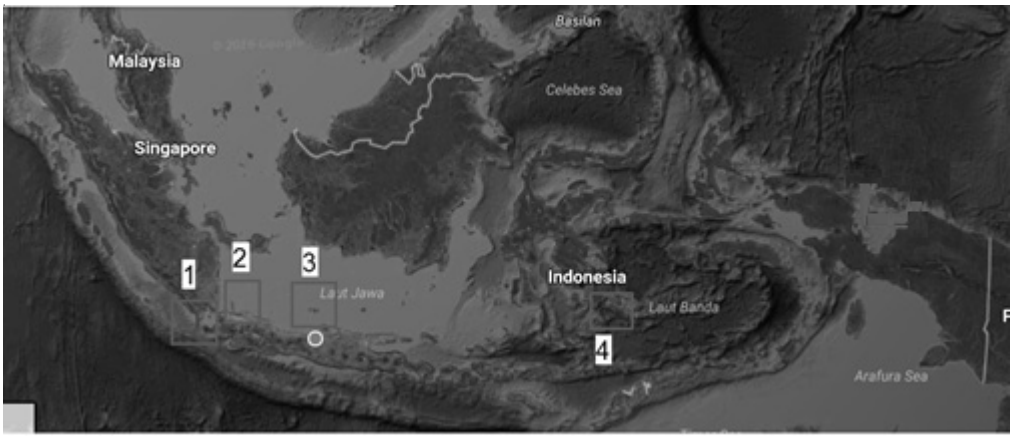

Fig. 1 Collection sites for sponge specimens: Lampung (1), Seribu Islands (2), Karimunjawa Islands (3), and Wakatobi Islands (4).

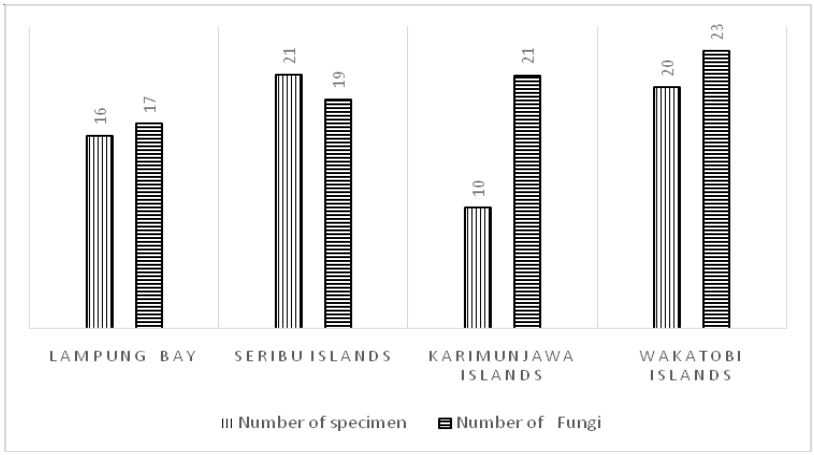

Fig. 2 The number of marine associated fungi from four different collection sites

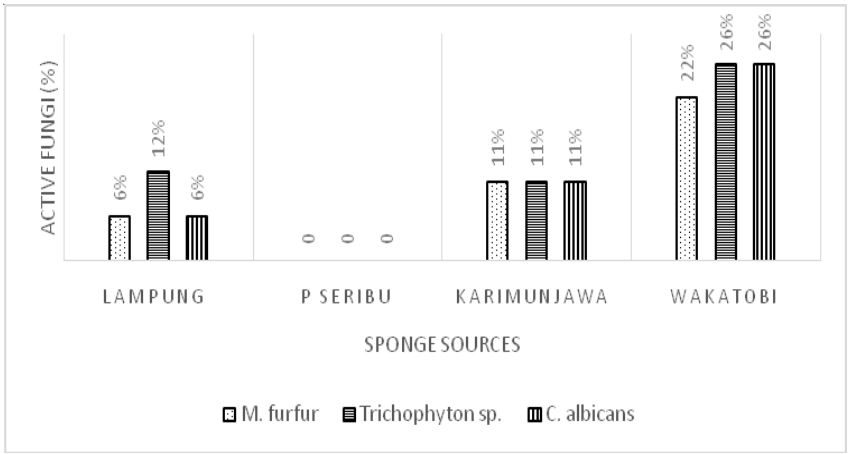

Fig. 3 The number of active isolates obtained from four different locations (percent).

The preliminary screening test on antifungal properties resulted in 10 active isolates (11.9%) from the total isolates. The Wakatobi isolates exhibited the highest number of the active isolates (60%) against the fungal pathogens, whereas the Karimunjawa and Lampung isolates both contributed 20% active isolate. The Seribu Island isolates did not show activity on theantifungal test.

Table 1. Inhibition zone presence fungal isolates against selected fungal pathogen by cross streak method against *M. furfur*, *Trichophyton* sp. and *C. albicans*

No.	Isolate code	*M. furfur*	*Trichophyton* sp.	*C. albicans*
1.	LB-18-1	-	-	+++
2.	LB-18-2	+++	++	++
3.	16-WK-1	++	+++	+++
4.	16-WK-2	++	++	-
5.	16-WK-6-1	++	+++	+++
6.	16-WK-12	+++	++	++
7.	16-WK-15-2	-	++	++
8.	16-WK-16-2	+++	+++	+++

Note: +++ (strong), ++ (moderate), + (weak), - (no activity)

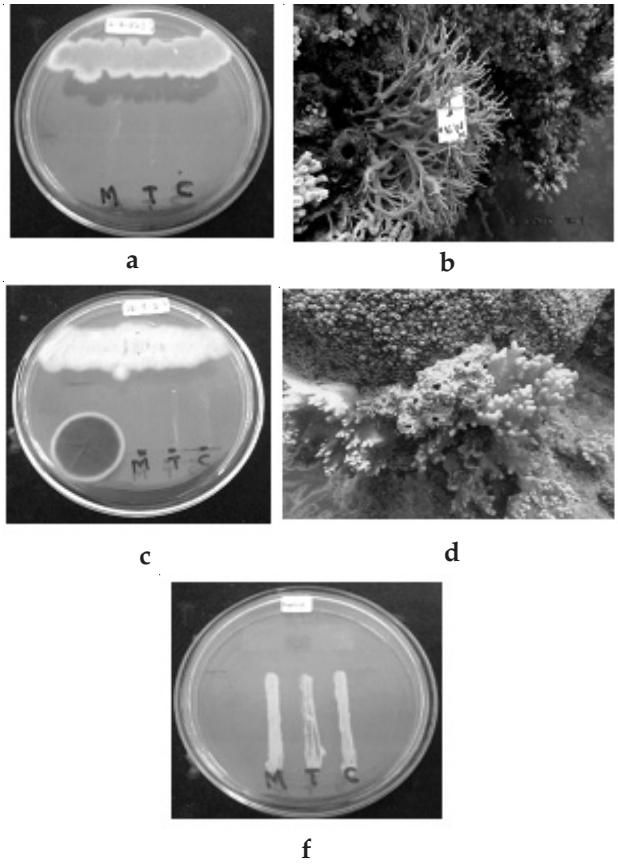

Fig. 4 The screening against the pathogenic fungi using cross streak method. a. Isolate of the sponge 16-WK-1 (b). c. Isolate of sponge LB-18 (d). e. The control of fungal pathogen (M= *M. furfur*, T=*Trichophyton* sp, C= *C. albicans*).

Fungal Characterization

The morphology of the isolates were observed directly and under a microscope for preliminary identification. Thus, the characterization also has been done under the microscope, an example as shown in Figure 5.

The macroscopic feature of L-11-2 shows-powdery-dark green spores with white mycelia that gradually turn to transparent. Under the microscope view, the hyphae have septae (Figure 5b), and the sporeis easily removed from the conidia resulting the solitary spore.

Table 2. The inhibition zone on the antifungal assay results of the isolate L-11-2 extract against *M. furfur*, *Trichophyton* sp. and *C. albicans*

No.	Concentration levels (µg/disc)	Diameter of inhibition zone (mm)		
		M. furfur	*Trichophyton* sp.	*C. albicans*
1.	500	8.0	10.5	7.0
2.	250	6.0	10.5	6.0
3.	100	7.5	10.0	3.5
4.	(+) control	17.5	15.0	14.5
5.	(-) control	0.0	0.0	0.0

- : no inhibition zone; (+) control: nystatin (30 µg/disc) , (-) control: ethyl acetate

Antifungal Test of the Active Isolates

After the primary screening of all the isolates, a total of 10 isolates were active as anantifungal. The isolate L-11-2 extract was then carried out for the secondary screening by using agar diffusion method against *Malassezia furfur*, *Trichophyton* sp. and *C. albicans*. The assay was carried out in the different concentration levels: 500, 250 and 100 µg/mL per disc. The results showed that the extract was active on antifungal test against all fungal pathogens represented by inhibition zones which are shown in Table 2.

DISCUSSION

Sponge Collection and Isolation of Marine Fungal Symbiont

Since the marine environment is a complex ecosystem, various interactions may occur between the living organisms and its habitat. A number of studies successfully revealed a diverse marine fungal association in various hosts such as sponges, fish, sediment and mangrove tree (Jones, 2011).

The result of this study may correlate with the difference of fungal populations that exist within

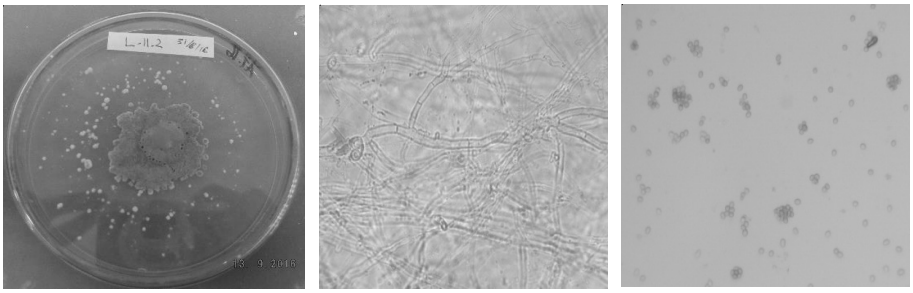

Fig. 5 Isolate L-11-2 on MEA plate (a), Hyphae of isolate L-11-2 having septae (arrows) with 400x magnification (b), and globus spores of L-11-2 with 400x magnification

different sponge species and the difference of the geographic locations. Fungal density and diversity in marine environment is affected by many factors such as temperature, salinity, nutrients, and competition among the microorganisms (Jones, 2000). Study on the biodiversity of marine fungi collected from three different sponges from two different locations describes that the species appear that are only found in one sponge species (sponge-specialist), found in more than one sponge sp. (sponge-associates) and found in all sponge sp. (sponge-generalists) (Jones, 2000). The number of associated-fungi in the sponge vary from 0-11 species (Gandhimathi *et al*., 2008; Li, 2009).

Screening for Antifungal Activity

Wakatobi Islands are the best resource of fungi isolates among the collection sites. Wakatobi Islands are located on "coral triangle center" that has high biodiversity of marine organisms (Wallace, 2011). In this area competition for space and food are very high. Therefore marine organisms have to produce bioactive compounds for their survival. Marine organisms produce uncommon bioactive secondary metabolites to adapt and survive in their habitat that are characterized by peculiar environment conditions (Bhakuni and Rawat, 2005).In nature, bioactive compounds produced by marine organisms were aimed to expel the predator, space competition, communication, hunting and as anti micro-organisms.

Interestingly, the isolates from Seribu Islands did not show the activity against the pathogenic fungi. Seribu Islands is located near the Jakarta City where the water contamination is relatively high. Heavy metals are one of a pollutants that contaminated the Jakarta bay such as Hg, Pb, Cd, Cr etc. (Haryati *et al*., 2013) and in turn it will flow to Seribu Islands. Sponges are well known as heavy metal accumulators (Rao *et al*., 2006) and are able to accumulate heavy metal in high concentration, and in turn, it will flow to Seribu Islands. Metal, oils, and chemical may act as elicitor compounds such that alter the production of secondary metabolites by microorganisms (Zhang, 2005). Presumably, the pollutant both in water and sediment in Seribu Island affect the production of bioactive compounds of the sponge-associated microorganisms.

Fungal Characterization

The investigations on sponge's fungal assemblage resulted in the isolation of various genera that were dominated by *Acremonium, Aspergillus, Fusarium, Penicillium, Phoma* and *Trichoderma* (Morrison, 2002; Li, 2009; Wiese *et al*., 2011). Considering that most of the marine fungi are belonging to mitosporic fungi (Morrison, 2002), the molecular-based approach should be used to complete the fungal identification.

Antifungal test

Some literature reported that marine-derived fungi have antifungal properties. Marine fungus *Penicillium cf. montanenseis* an associated fungus of marine sponge *Xetospongiaexigua* that produce xestolactone B compound. This compound has antifungal activity against *C. albicans* (Edrada *et al*., 2002).

CONCLUSION

The marine habitat is housing the untapped diversity of fungi that has not yet been fully explored. This study revealed 10 sponge-associated fungi from different marine environments in Indonesia that showed antifungal activity against pathogenic dermatophytes.In the near future, bioactive compounds from the active isolates need to be isolated and further purified.

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