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The effect of culture media on the number and bioactivity of marine invertebrates associated fungi

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Abstract. *Trianto A, Radjasa OK, Sibero MT, Sabdono A, Haryanti D, Zilullah WOM, Syanindyta AR, Bahry MS, Widiananto PA, Helmi M, Armono HD, Supriadi, Igarashi Y. 2020. The effect of culture media on the number and bioactivity of marine invertebrates associated fungi. Biodiversitas 21: 407-412.* Marine ecosystem is rich with microorganisms such as bacteria and fungi either as free-living or in association with macro-organisms. Marine invertebrates provide suitable habitats for fungi by supplying space, food, and other chemicals stuff that in some cases is a reciprocal relationship or called mutualism symbiotic. Some marine invertebrates have interesting activities that are useful for human life such as anticancer, antifungal, and antibacterial. Many reports indicated that the fungal growth and their production of bioactive compounds were highly affected by the media or nutrition. In order to understand the effect of media on the number and bioactivity of the isolates, we collected the samples of marine invertebrates from two locations in Makassar. Invertebrate specimens were collected by hand during SCUBA diving at 3-10 m depths. The fungi were isolated by tapping method either on potato dextrose agar (PDA) or poor marine agar (PMA). The samples were collected from the Samalona water as much as 16 specimens that provided 30 and 18 fungal isolates on PDA and PMA, respectively, while, from the Barrang Cadi water, a total 14 specimens were collected to provide 12 and 3 isolates on PDA and PMA, respectively. All fungi from PMA inhibited the *V. harveyi*, *V. vulnificus*, and *V. parahaemolyticus* with weak, medium, and strong activities, while, the isolates from PDA were mostly not active against the Vibrios. Based on the molecular analyses, the active isolates were identified as *Aspergillus flavus*, *A. oryzae*, *A. aculeatus*, *Talaromyces minioluteus*, *Hypocrea jecorina*, *Gliomastix murorum*, *Myrothecium inundatum*, and *Curvularia avinis*. In conclusion, the isolates from PMA showed higher potential as source of antivibrio substances.

Keywords: Sponge, tunicate, nudibranch, fungi, vibrio

INTRODUCTION

Marine microorganisms are widely studied as source of secondary metabolites that are useful for human life (Carroll et al. 2019; Pham et al. 2019). Among all marine microorganisms, fungi get a special concern due to its productivity in producing novel bioactive compounds (Tarman et al. 2011; Zhou et al. 2014; Lindequist 2016). One of bioactive compounds from marine fungi is Plinabulin which is isolated from *Aspergillus* sp. It is being investigated by Food and Drug Administration (FDA) to be applied for cancer therapy (Pereira 2019). Furthermore, plenty of bioactive compounds from marine fungi are isolated every year in order to obtain new drugs for human health (Imhoff 2016; Lindequist 2016). Marine fungi have also been reported that produce antimicrobial compounds such as isaridins, cristatumins, and stachyins (Xu et al. 2015). The capability to producing novel bioactive compounds leads to massive isolation of marine fungi from various hosts and locations.

Indonesia's marine ecosystems are considered to host enormous untapped marine fungi. Marine fungi are commonly found as a free-swimming organism or living in association with other micro-organisms such as sponge, coral, and tunicate (Gradinger 2016; Grossart et al. 2016; Hassett and Chen et al. 2018; Sibero et al. 2018; Xu et al. 2018). Therefore, Spermonde Archipelago in Makassar, South Sulawesi, Indonesia is suggested as one of prospective locations that harbor marine fungi due to its diversity of marine invertebrates (De Voogd et al. 2006;

Litaay et al. 2018). Several genera such as *Aspergillus*, *Cladosporium*, *Daldinia*, *Eutypella*, *Fusarium*, *Lasiodiplodia*, *Trichoderma* were previously isolated as invertebrate-associated fungi from Indonesia (Tarman et al. 2012; Trianto et al. 2018; Sibero et al. 2019). These fungi also performed decent antibacterial activity against pathogenic bacteria.

In addition, most of studies were applied in a rich-nutrient medium to isolate marine fungi such as potato dextrose agar (PDA), Sabouraud dextrose agar (SDA), cornmeal agar (CMA), malt extract agar (MEA) and yeast malt agar (YMA) (Trianto et al. 2017; Chen et al. 2018; Sibero et al. 2018). The influence of isolation medium to the diversity of cultivable-marine fungi had been reported, however, study of the influence of isolation medium to the antibacterial activity of the cultivable-marine fungi is rarely done. Therefore, the purpose of this study was to investigate the effect of standard and poor media on the number and anti-vibrio activity of cultivable invertebrate-associated fungi from Spermonde Islands in Makassar, Indonesia.

MATERIALS AND METHODS

Sample materials

Marine invertebrates such as sponge, coral, tunicate, and nudibranch were collected around Samalona and Barrang Caddi, Spermonde Islands, Makassar, Indonesia (Figure 1) by SCUBA at 3-10 m depth. All samples were documented under and above water. Samples were kept inside a sterilized zip lock then transferred for fungal isolations.

Fungal isolation

A rich nutrient medium, potato dextrose agar (PDA, (HiMedia) and a poor marine agar (PMA) were used to isolate invertebrate-associated fungi. Poor nutrient agar (PMA) has consisted of agar and marine water without any additional nutrients. Fungal isolation was performed according to Sibero et al. (2019) using tapping method. Samples were cleaned using a running sterilized marine water and alcohol 70%. The surface of samples was discarded to remove surface contamination. Further, samples were cut into approx. 1 cm x 1 cm and put onto isolation media then incubated at room temperature (27 °C) until fungal growth was identified. During isolation, environmental control was prepared using PDA and PMA. All mycelia growing surrounding samples were transferred onto new media as a pure culture. Each fungus from isolation media was compared to the fungi from environmental control and the distinct isolates were confirmed as associated fungi then used for the further steps.

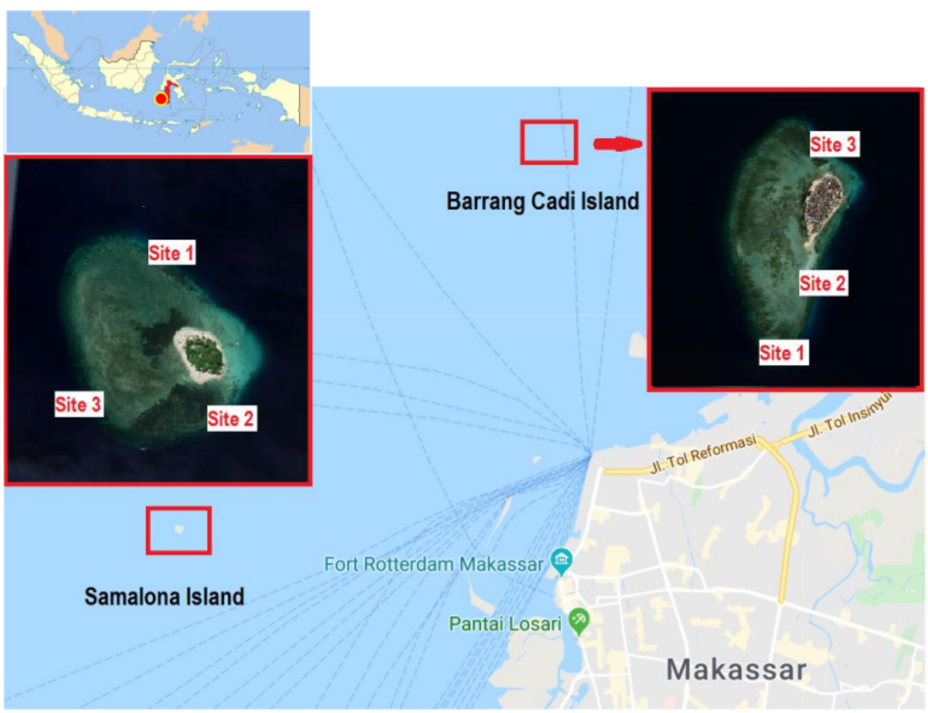


Figure 1. The collection sites of the marine invertebrates in Samalona and Barrang Caddi, Spermonde Islands, Makassar, Indonesia

Antibacterial screening

Antibacterial activity of all isolates was confirmed using agar plug method (Balouiri, Sadiki and Ibnsouda 2016; Sibero et al. 2019) against vibriosis agents such as *Vibrio harveyi*, *V. parahaemolyticus*, and *V. vulnificus*. The pathogens were recultured on nutrient agar medium for 24 h at 32 °C whereas, the pure isolates were cultivated on agar medium for 7 days at room temperature (27 °C) before performed the assay. In antibacterial assay, the pathogens were diluted into nutrient broth and its density was set up to 0.5 McFarland standards. Testing agar media were prepared by inoculating the pathogen solution onto the surface of nutrient agar media using a sterilized cotton swab. Then, approximately 1 cm^2 of fungal culture with its agar were cut and plugged onto the inoculated testing agar. The testing media were incubated at 32 °C to maximize the pathogen's growth for 24 h. Further, the formation of clear zone around the agar plugs indicated the antibacterial activity of the prospective isolates.

Fungal identification

Molecular identification of prospective isolates was carried out by amplifying the internal transcribed spacer (ITS) as the finger print region for fungal barcoding using polymerase chain reaction (PCR) thermal cycler. The ingredient of PCR mix was 12.5 µL of GoTaq Green Master mix from Promega Corporation, 1 µL of ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) as forward primer, 1 µL of ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) as reverse primer from Macrogen, 9.5 µL of ddH_2O and 1 µL of DNA template Trianto et al. (2018) and Sibero et al. (2019). PCR condition was denaturation at 95°C for 1 min; 34 cycles of denaturation at 95 °C for 3 min, annealing at 56.1 °C for 1 min, extension at 72 °C for 1 min; final extension at 72 °C for 7 min and cooling at 4°C until recovery of the samples. The PCR product was sent to Genetika Science for sequencing. The sequence results were compared to GenBank database in The National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST). The phylogenetic tree was reconstructed using MEGA 6.

RESULTS AND DISCUSSION

Samalona and Barrang Caddi Islands in Spermonde archipelago, Makassar were chosen as research sites due to the diversity of marine invertebrates. Early study of de Voogd et al. (De Voogd et al. 2006) reported that the sponge diversity in Spermonde archipelago which was dominated by *Amphimedon*, *Callyspongia*, *Chalinula*, *Clathria*, *Haliclona*, *Hyrtios*, and *Petrosia*. Further study reported the finding of more genus and species such as *Aaptos*, *Agelas*, *Aplysina*, *Cliona*, *Dysdea*, *Haliclona*, and *Xestospongia* in these areas (Haris et al. 2014). In addition, another invertebrate such as *ascidian* has been well studied in the same location by Litaay et al. (Litaay 2018; Litaay et al. 2018). The reports mentioned the species diversity of the ascidian in Spermonde archipelago was influenced by

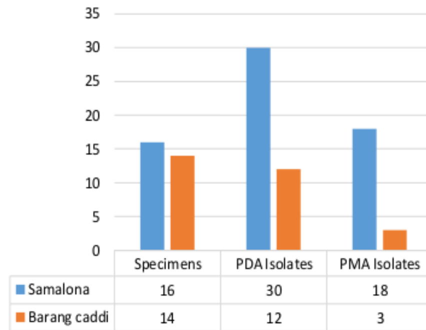


Figure 2. Invertebrate-associated fungi from Samalona and Barang Caddi, Spermonde Archipelago, South Sulawesi, Indonesia in different isolation media

the environmental conditions to support its survival ability. Moreover, *Polycarpa* and *Didemnum* were the most dominant ascidian. This study successfully collected 30 marine invertebrates from Samalona and Barrang Caddi Islands that were expected to harbor associated fungi, therefore fungal isolation could be performed. Figure 2 shows the abundance of the fungal isolates that grown in PDA and PMA media.

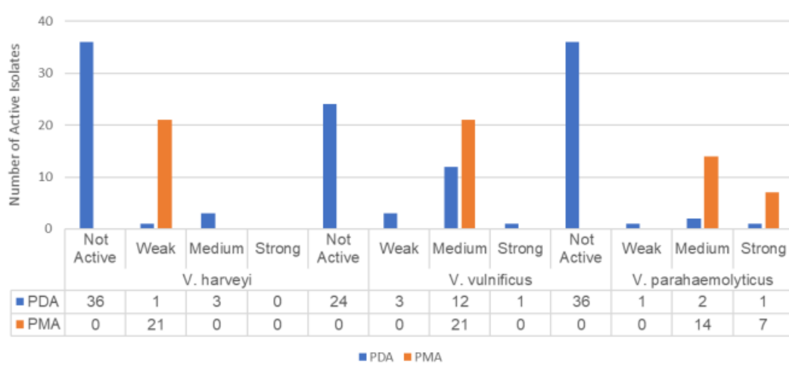
Several studies used different cultivation medium to obtain more diverse fungal species. Xu et al. (2018a) performed fungal isolation from deep-sea sediment with 6 cultivation media, for instance, Malt Extract Agar (MEA), Czapek Dox Agar (CDA), Corn Meal Agar (CMA), Sabaurauds dextrose agar (SDA), Yeast extract-malt Agar (YMA) and Potato Dextrose Agar (PDA). The study found that YMA was the best medium because it resulted in 30 isolates with 9 species. On the other hand, another study stated that PDA resulted in higher fungal abundance than other media such as MEA, CDA, SDA, CMA, PDA, YMA, Martin medium (MAR), and Murashige and Skoog medium (MSA) that were used to isolate scleractinian coral-associated fungi (Xu et al. 2018b). This study utilized PDA as the representatives of rich nutrient media because previous studies indicated this media is one of the proposed media for fungal isolation from marine environment. As expected, as a rich nutrient medium, PDA harbored number of isolate because it provided the nutrients that are needed by the fungi to grow and sporulate. PDA media gave 42 isolates whereas only 21 isolates were isolated from PMA. Certain substances such as carbon source, nitrogen source, and trace minerals are noted very essentially to influence the abundance of the cultivable fungi during the isolation (Sharma and Pandey 2010; Muggia et al. 2017). Furthermore, a similar study which was done by Bovio et al. (2019) reported a similar result. The poor marine agar (PMA) could not provide any nutrients that supported fungal growth, therefore, only a few fungal taxa can grow on it. In addition, aside of nutrient in cultivation media, several factors that influence the number of fungal taxa were isolation technique, tissue structure, the host, habitat, incubation temperature, and the metabolite that was produced by the host (Henríquez et al. 2014; Calabon et al. 2018; Xu et al. 2018; Bovio et al. 2019; Sibero et al. 2019). In order to obtain prospective isolate which produces antibacterial compound, a screening using plug media against vibriosis agent was performed. The result of antibacterial screening activity is shown in Figure 3.

Plug method was carried out to screen the antibacterial potential of all isolates. Basically, the plug method relies on the metabolites that are secreted into the cultivation agar medium during the fungal growth, afterward metabolites scatter onto the test agar which has been inoculated by the pathogen. Künzler (2018) stated that fungi are used to secrete effectors to inhibit or kill microbial competitors, while the effectors against metazoan predators are produced and stored within the cell. The production of antibacterial substances will be expressed by the presence of inhibition zone because the bacteria around the agar plug are killed by the secreted metabolite (Balouiri et al. 2016; Sibero et al. 2018, 2019). Table 1 shows that plenty of fungi exhibited antibacterial property against vibriosis agents. The antibacterial activity of the isolates was distinguished as bactericidal and bacteriostatic. Bactericidal activity is indicated by the formation of permanent inhibition zone because the metabolite kills the bacteria, thus the inhibition zone is not recovered by the bacterial growth. Vice versa, bacteriostatic activity only inhibited the growth of the pathogens, therefore the diameter of inhibition zone will be decreased by the time (Nemeth et al. 2015). Interestingly, fungi isolated using PDA were not as potent as fungi isolated using PMA (Figure 3). Among 40 isolates from rich nutrient media, only 4 isolates performed antibacterial against *V. harveyi*, 4 isolates inhibited *V. parahaemolyticus* and 16 isolates for *V. vulnificus*, whereas all isolates from PMA exhibited antibacterial activity against all vibriosis agents. It is never been reported the fungal secondary metabolite was produced in a PMA which contained only seawater and agar. This fact gives another point of view on fungal biological activity.

The ability of all isolates from PMA might be induced by the nutrition scarcity in the medium (Demain 1998; Ruiz et al. 2010). Nutrient availability such as carbon and nitrogen influence the sporulation process and its natural product productions. Several works have proven that the source of carbon and nitrogen greatly impacts the antibacterial activity of fungi (Bhattacharyya and Jha 2011; Jain and Gupta 2012; Rani and Jain 2017). This study obtained an interesting result that fungi producing metabolite on PMA strongly inhibited vibriosis agents. However, further study is strongly needed to understand the produced metabolites.

Molecular identification

The molecular analyses as shown in Figure 4, reveals that the most active isolates belong to the genus Aspergillus i.e. *A. flavus* (3 isolates), *A. orizae* (1 isolate), and *A. aculetus* (1 isolate), while other identified isolates are *Talaromyces minioluteus* (1), *Hypocrea jecorina* (1), *Gliomastix murorum*, *Myrothecium inundatum* (1), and *Curvularia affinis* (1). Aspergillus is a genus consisting of a few hundred mould species found in various climates worldwide. *Aspergillus flavus* is a saprotrophic and pathogenic [1] fungus with a cosmopolitan distribution. It is best known for its colonization on cereal grains, legumes, and tree nuts. Postharvest rot typically develops during harvest, storage, and/or transit. *A. flavus* infections can occur while hosts are still in the field (preharvest) without any symptoms (dormancy) until postharvest storage and/or transport. Many strains of Aspergillus produce toxic compounds known as mycotoxins in high quantities. A noncarcinogenic and aflatoxin-free *Aspergillus flavus* strain AF36 is used as an active ingredient in pesticides which was used as commercial biocontrol in cotton and corn to reduce aflatoxin exposure.



	Not Active	Weak	Medium	Strong	Not Active	Weak	Medium	Strong	Not Active	Weak	Medium	Strong
	V. harveyi				V. vulnificus				V. parahaemolyticus			
PDA	36	1	3	0	24	3	12	1	36	1	2	1
PMA	0	21	0	0	0	0	21	0	0	0	14	7

Figure 3. The active isolates from PDA media and PMA media against the vibrio

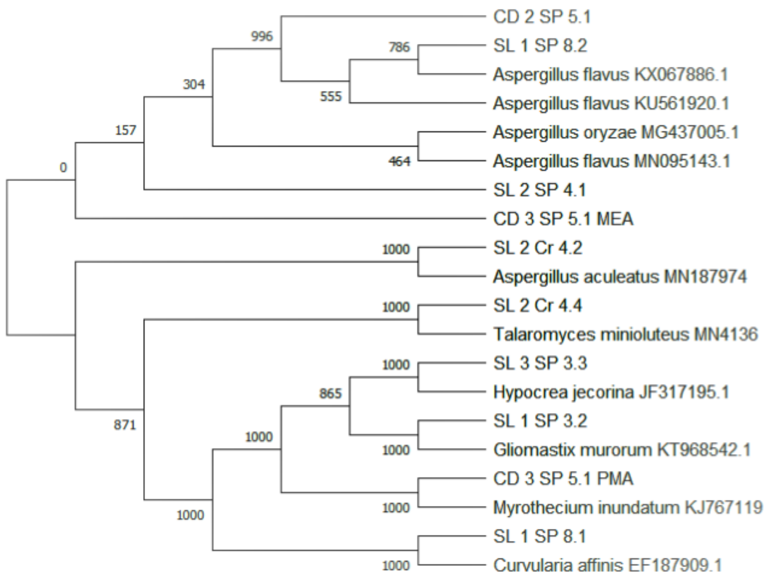


Figure 4. Phylogenetic tree of active fungal isolates from Samalona dan Barrang Cadi Islands, Spermonde Islands, Makassar, Indonesia

Aspergillus oryzae is a filamentous fungus used in Japan to ferment soybeans for making soy sauce and fermented bean paste (including miso and also to saccharify rice, other grains, and potatoes in the making of alcoholic beverages such as sake and shōchū. *A. oryzae* is also used for the production of rice vinegar and for production of resveratrol from its glucoside piceid (Wang et al. 2007).

A. aculeatus belongs to the group of black Aspergilli which are important industrial workhorses. *A. aculeatus* is considered to be a ubiquitous species that could be usually isolated from rotting fruits and soil. Modern biochemical and molecular identification techniques are helpful in the identification of Aspergillus isolates, such as black-spored Aspergillus species may have significant variations in their morphological and physiological characteristics. Aspergillus can rapidly degrade cell walls of plants they infect, and isolates of *A. aculeatus* have been used to produce a number of important industrial enzymes, including cellulases, hemicellulases, and proteases. These by-products are broadly used in the food and feed industries. Due to its industrial value, the biochemical and catalytic properties of several hydrolases from *A. aculeatus* have been extensively studied. Also, structural studies using X-ray crystallography have been carried out on several polysaccharide degrading enzymes from *Aspergillus aculeatus.*

Talaromyces minioluteus is an important fungal genus because of its ubiquity which was isolated from soil, plants, sponges, and foods. Some of the species are heat resistant. Some of the species are famous because of their enzymes applicable in the synthesis of saccharides, preparation of chiral building blocks or biotransformations, and for its application in pest biocontrol. Many of its species are used in food and agricultural production (Jie et al. 2016). *Hypocrea jecorina*, The pantropical ascomycete *Hypocrea jecorina* (anamorph *Trichoderma reesei*) is known as an industrial producer of cellulolytic enzymes (Lynd et al. 2002). The mechanism, which H. induces jecorina cellulases has remained enigmatic, especially since cellulases are only formed upon induction but the natural inducer (=cellulose) cannot pass the cell wall and plasma membrane and thus cannot enter the cell (Schmoll and Kubicek 2005). *Curvularia affinis* is an ecologically and economically important genus and is known as an anamorph of Cochliobolus Drechs. Pleosporales (class Dothideomycetes, Ascomycota). The approximately 54 species are included in the genus and are usually known as subtropical and tropical facultative parasites on herbaceous plants (Yanagihara et al. 2010).

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