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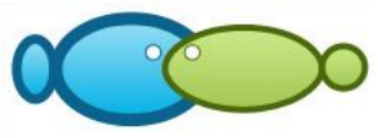

Coral-associated fungi as a natural inhibitor for treatment of multidrug-resistant pathogens

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Abstract. Researches on bioactive compounds from hard coral-associated fungi (HCAF) in Indonesia have been less documented than bacterial associated part. This is especially promising when it comes to find a solution to the problem of antibiotic-resistant bacteria. This study's aim was to determine the inhibitory potential of HCAF against several selected pathogenic bacterial strains. The discovery of novel bioactive compounds generally involves three major steps. The first step is the isolation of hard coral samples in an appropriate media, followed by an antimicrobial assay of an HCAF against various microbial pathogens, and lastly the identification of potential microbes. Assay values for the compounds were determined against the following multidrug-resistant (MDR) bacteria, namely *Staphylococcus haemolyticus, Staphylococcus aureus*, *Pseudomonas aeruginosa, Klebsiella pneumoniae, Enterobacter aerogenes, Acinetobacter baumannii, Enterobacter cloacea* complex and *Escherichia coli*. This study successfully identified HCAF as having potential as an antibacterial compound. Inhibitory activity on the growth of mentioned MDR was visible on 18 out of 38 fungi. As a result, the potential these novel antibacterial agents with activity against MDR bacteria is highlighted. The study of HCAF can serve as a powerful strategy for the discovery of novel antibiotics against human pathogens.
Key Words: marine invertebrate, inhibitory activity, antibiotic-resistant bacteria, marine derived-fungi.

Introduction. The threats from drug-resistant human pathogens reported by the CDC (2013) and the urgency for new antibiotics stated by the WHO (2017) require prompt and sustained action to combat infectious diseases and also to decrease health problems. Alternative solutions to multidrug-resistant (MDR) infections will help reduce drug-resistant infection per year, excess hospitalizations, deaths, and excess medical costs per year around the world (CDC 2013). The above lists were drawn up in a bid to guide and promote research and development of new compounds as candidate drugs.

The biodiversity of coral reefs and their secondary metabolites could be a source of bioactive substances useful in modelling compounds for drugs (Radjasa et al 2008). Many marine-derived natural compounds with anti-microbial potential are produced by marine organisms (Radjasa et al 2013). Marine-derived fungi have proven to be a promising source of structurally novel and biologically active secondary metabolites that have become a significant resource for drug discovery (Blunt et al 2012; Cristianawati et al 2019). Some related research of bioactive compound produced by coral are: carijoside A, 1 isolated from *Carijoa* sp. (Liu et al 2010), anti-biofilm compounds from coral-associated actinomycete (Bakkiyaraj & Karutha Pandian 2010), anti-quorum sensing from bacterial extract (Ma et al 2018) and antibiofilm activity (Song et al 2018) from coral-associated bacteria.

The findings of our research are of public concern and there has been little attention given to the widening gap between the overexploitation of marine organisms and ethical implications. In addition, no major therapeutic drugs originating from a single bioactive marine compound active against multiple MDR bacteria has yet been developed. Due to the urgency, we have done further research into bioactive compounds from marine hard coral-associated fungi (HCAF) collected from Panjang Island, North Java Sea, Indonesia, potentially discovering a compound with anti-MDR pathogen properties, without damaging the host, and to identify the isolates that show anti-MDR pathogen potential using a molecular approach.

Material and Method

Sampling of hard coral and isolation of associated fungi. A total of 8 hard coral samples were collected from Panjang Island, Indonesia (Figure 1). The hard coral colonies were collected by snorkeling and were put into sterile plastic bags (Whirl-Pak, Nasco, USA) containing 50 mL of seawater. The samples were transported in a cooling box at temperatures below 4°C for three hours before further processing in the laboratory according to Trianto et al (2017). HCAF were isolated with the same method as used by Strobel & Daisy (2003). Using a sterile scalpel, the living tissue of the coral was cut in pieces of approximately 0.1 cm^3 in size and sprayed three times with sterilized seawater. These pieces were then rinsed for surface sterilization with sterilized seawater and 70% EtOH. From each hard coral, coral tissues were applied to Malt Extract Agar (MEA) medium (HiMedia™, Mumbai, India) containing chloramphenicol antibiotic (100 mg mL^{-1}) and were then incubated in an incubator where they remained for 7 days at 28°C. Morphologically different colonies of fungi were purified using a sterile loop and placed into a new plate medium.

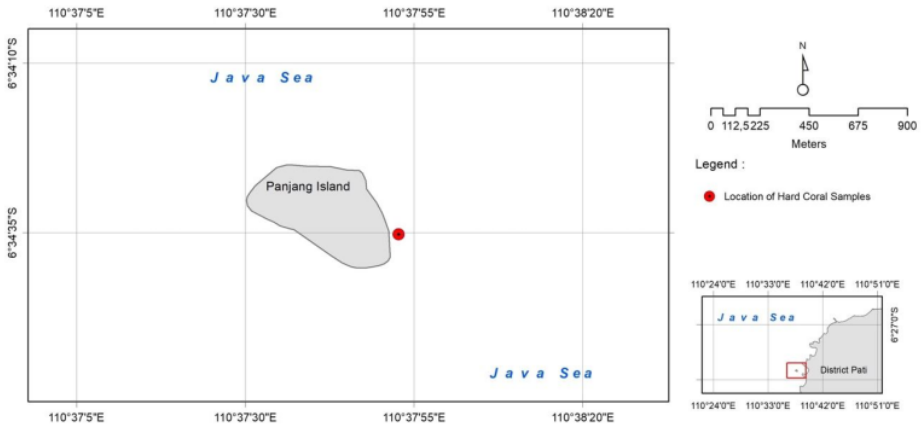


Figure 1. Sampling site for the collection of hard coral samples from Panjang Island, Indonesia. Source: GeoEye-1 satellite.

The MDR pathogen for antibacterial testing. The MDR pathogens were obtained from the culture collection of Dr. Kariadi Hospital Semarang, Central Java, Indonesia. These bacteria, well-recognized nosocomial pathogens belonging to the *Pseudomonas aeruginosa, Klebsiella pneumoniae, Enterobacter aerogenes, Acinetobacter baumannii, Enterobacter cloacae* complex*, Staphylococcus haemolyticus, Staphylococcus aureus* and *Escherichia coli,* were subjected to antibacterial testing. The fresh and pure cultures of MDR pathogens were diluted to a suspension equivalent to 0.5 McFarland standard.

Fungi preparation for antibacterial testing. A total of 34 coral-associated fungi were collected. Morphologically different colonies were cut in tablet pieces of approximately Ø 8 mm in size using a sterile loop under a fume hood to avoid contamination.

Screening for antagonistic activity of hard coral-associated fungi. Antimicrobial activity was determined following a method proposed by Rahaweman et al (2016), Sibero et al (2017), and Sabdaningsih et al (2019). The suspension of each MDR pathogen was spread over the entire area of a Mueller Hinton (MH) agar (Oxoid™, Basingstoke, UK) plate using a sterile cotton swab. Single tablets of fungi were placed on the inoculated MH agar plate, ensuring sufficient space between individual tablets to allow for proper measurement of inhibition zones. A tablet of MEA medium (Ø 8 mm) without any fungi was used as a negative control. Chloramphenicol antibiotic (30 µg, Ø 8 mm Oxoid™) was used in the assay as positive control. The plates were incubated at 35°C for 24 hours. The presence of a clear zone indicated antibacterial activity.

DNA extraction of potentially interesting fungal isolates. In total 18 active fungal isolates were prepared for further analyses. DNA from mycelia was extracted using a Chelex extraction method (Qiu et al 2005; Cristianawati et al 2017).

PCR amplification and sequencing of 18s rRNA gene fragments. The internal transcribed spacer (ITS) region was amplified by using universal primer forward ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') (Macrogen Inc., Seoul, Korea) and primer reverse ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Macrogen) (Sabdaningsih et al 2017). These primers were used to obtain 400-800 bp rDNA fragments for sequencing purposes. A polymerase chain reaction (PCR) was carried out in a thermal cycler (T100™ Thermal Cycler, Bio-Rad Laboratories, California, USA). PCR was performed with 25 µL volumes consisting of 12.5 µL of GoTaq® Green Master mix (Promega, Madison, USA), 1 µL of each primer, 9.5 µL of nuclease-free water and 1 µL of extracted DNA template from the HCAF. PCR cycles were preheated at 95°C for 5 minutes, followed by 32 cycles of initial denaturation at 95°C for 1 minute, 56.4°C for 1 minute and 72°C for 1 minute followed by a final elongation step (7 min at 72°C).

Visualisation of PCR products. The amplified PCR products in 3 µL were separated by electrophoresis (MultiSUB Mini, Clever Scientific©, Warwickshire, United Kingdom) on 1% agarose gel stained with ethidium bromide. The Geneaid 100 bp DNA Ladder (Geneaid Biotech Ltd, New Taipei City, Taiwan) was used in 3 µL to ensure quick and easy determination of electrophoresis results. The run was performed in 1× TAE electrophoresis buffer for 30 minutes and a constant 100 V. The separated fragments on the agarose gel were visualized under UV-light (UVIdoc HD2, UVITEC Cambridge, England, United Kingdom).

Results and Discussion

Bioprospecting of hard coral-associated fungi. A total of 8 marine hard corals were collected from sampling locations along the Panjang Island, Indonesia (Figure 2). The previous research refer that the coral reefs of Indonesia are among the most diverse reefs in the world (Mora et al 2003).

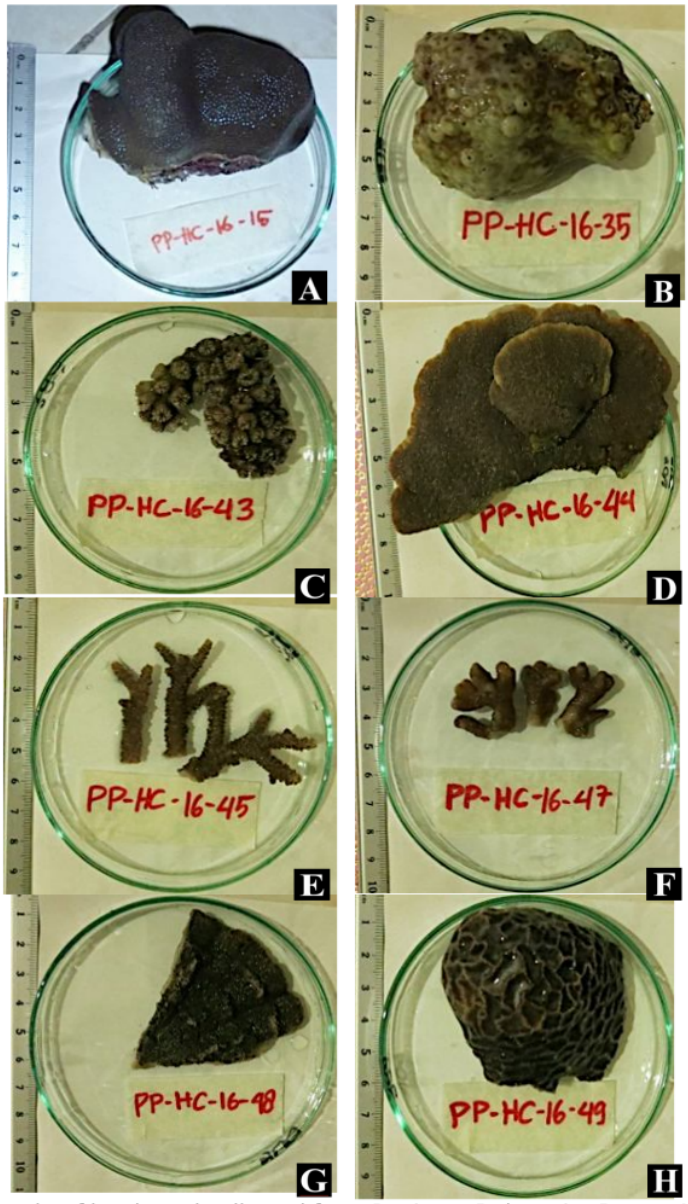


Figure 2. Sample of hard coral collected from Panjang, Indonesia. Image A, *Goniastrea* sp.; B, *Favia* sp.; C, *Caulastrea* sp; D, *Montipora* sp.; E, *Acropora* sp.; F, *Palauastrea* sp.; G, *Pachyseries* sp.; H, *Pavona* sp.

The samples were identified by using the book of Veron (2000) and software Coral ID (Budd et al 2012; Huang et al 2014). Genera of 8 different corals are presented in Table 1.

Table 1

Identification of the collected hard coral host producing potentially interesting fungal from Panjang Island, Indonesia

Sample ID	*Proposed species*	*Key identification**
PP-HC-16-15	*Goniastrea* sp.	Colonies massive, thick encrusting or columnar. Corallites round tending sub-meandroid (4-6 mm). Septa are fine and give walls a neat/smooth regular appearance.
PP-HC-16-35	*Favia* sp.	Colonies massive, domed/thick encrusting. Corallites form cones/tubes with separate walls (8-20 mm). Septa and costae well developed with fine to medium teeth.
PP-HC-16-43	*Caulastrea* sp.	Colonies made of single/short meandering corallites on stalks. Corallites (8-15 mm). Regular, bold septocostae visible though tissue on outside of corallite. Less spikey septa than small *Lobophyllia* species.
PP-HC-16-44	*Montipora* sp.	Delicate vases and tiered plates. Very small (0.1 mm) poorly defined corallites set among skeletal structures. Slightly smaller corallites and less defined corallite walls than encrusting *Porites*, which do not form vases.
PP-HC-16-45	*Acropora* sp.	Colonies branching, bushy/plate-like. An axial corallite at branch tips is surrounded by radial corallite.
PP-HC-16-47	*Palauastrea* sp.	Colonies have blunt finger-like branches that do not taper. Corallites very small (1 mm) with wagon wheel-like central spike. Uncommon coral.
PP-HC-16-48	*Pachyseries* sp.	Thin plates and fronds with corallites on one side only. Valleys (~5 mm) with beaded centreline. Septa form fine regular ribs. Distinctive.
PP-HC-16-49	*Pavona* sp.	Septocostae flow between corallites with indistinct walls. Corallites group in valleys, separated by sharp ridges. Septocostae better defined and not granular compared to *Coscinaraea* and septocostae coarser and inter-valley ridges sharper than *Leptoseris*.

*Identification based on Veron (2000); PP-HC-16 -n: Panjang Island - hard coral- Year 2016- Sample n.

Isolation and screening of antibacterial activity. From the collected hard coral specimens, 34 fungal isolates were obtained. The isolates were tested against 8 MDR pathogenic bacteria that resulted in 18 active isolates, as shown in Table 2.

Table 2

The abundance diversity of hard coral associated fungi

Proposed species	*N-associated fungi*	*Potentially interesting fungal*
Goniastrea sp.	7	FHP 2, FHP 3, FHP 5
Favia sp.	12	FHP 7A, FHP 7C, FHP 8, FHP 11, FHP 6, FHP 15, FHP 16, FHP 18, FHP 24
Caulastrea sp	2	FHP 25
Montipora sp.	1	-
Acropora sp.	4	FHP 21A, FHP 21B, FHP 34
Palauastrea sp.	1	FHP 38
Pachyseries sp.	5	FHP 37
Pavona sp.	2	-
Total	34	18 isolates

Note: FHP (Fungi Hard coral Panjang Island).

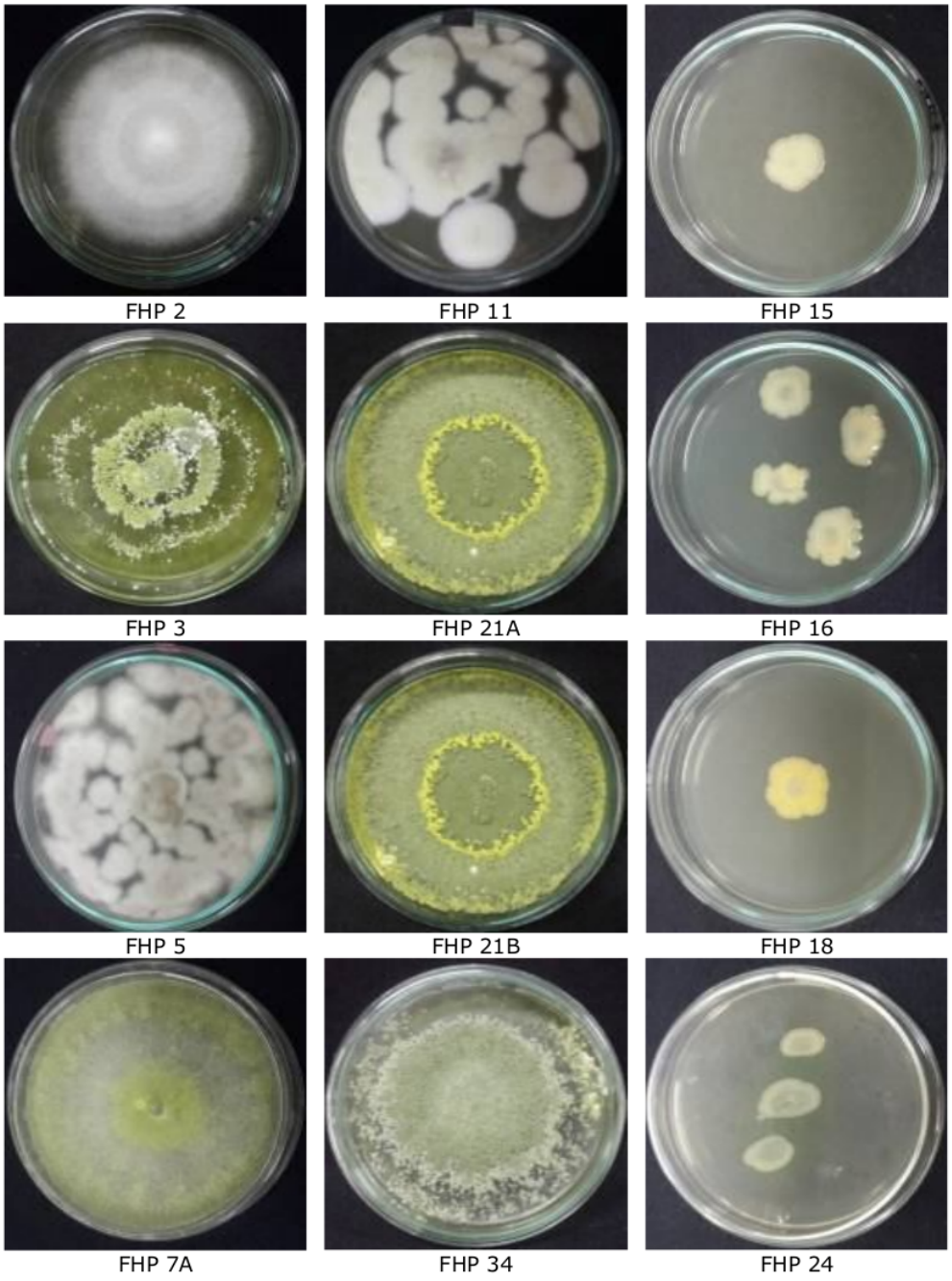
Screening test. The oceans are the sources of a large group of structurally unique natural products that are mainly accumulated in marine macrobes such as invertebrates (e.g. coral, sponges, softcorals, tunicates) and algae. Several of these secondary metabolites have pronounced pharmacological activities (Blunt et al 2010). Fungal isolates (n = 34) were successfully isolated from 8 marine hard corals. Screening of HCAF against MDR pathogenic bacteria resulted in a total of 18 active isolates that at least inhibited the growth of one pathogenic bacterium (Table 3). The morphology of potentially interesting fungi are displayed in Figure 3.

Table 3

Antibacterial test of hard coral associated fungi against MDR pathogens

Isolate	*Antibacterial activity (mm)*							
	Pa	*Kp*	*Ea*	*Ab*	*Ecc*	*Sh*	*Sa*	*Ec*
FHP 2	-	-	6.52±0.64	-	-	-	-	2.95±1.09
FHP 3	6.70±0.27	-	7.90±0.00	-	-	14.87±2.76	4.30±00.00	3.65±0.288
FHP 5	-	-	-	-	-	1.82±1.141		
FHP 7A	-	-	7.07±0.45	-	-	6.00±00.00	3.87±0.75	-
FHP 7C	-	-	-	-	-	16.95±1.45	9.35±1.61	-
FHP 8	-	2.75±0.50	3.72±0.44	-	-	-	-	-
FHP 11	5.52±0.87	-	-	-	-	12.83±1.38	-	-
FHP 21A	-	-	9.35±0.43	-	-	13.45±0.98	-	-
FHP 21B	-	-	9.52±0.46	-	-	13.45±0.98	-	-
FHP 34	11.35±0.70	-	6.88±1.07	-	-	13.65±0.40	7.7±00.00	0.80±0.69
FHP 37	-	-	-	-	-	10.70±0.00	-	-
FHP 6	-	-	-	-	28.00±1.82	-	-	-
FHP 15	-	-	-	-	23.57±2.11	-	-	-
FHP 16	-	-	-	-	11.20±1.36	-	-	-
FHP 18	-	-	-	25.90±4.83	-	-	-	-
FHP 24	-	-	-	-	25.00±00.00	-	-	-
FHP 25	-	-	-	-	25.00±00.00	-	-	-
FHP 38	-	0.80±0.00	-	-	6.35±1.21	6.25±0.65	-	5.50±0.57

Note: (-): Did not exhibit antibacterial activity. Data were average±standard deviation. Antibacterial screening with following MDR pathogens: Pa (*P. aeruginosa*), Kp (*K. pneumoniae*), Ea (*E. aerogenes*), Ab (*A. baumannii*), Ecc (*E. cloacae* complex), Sh (*S. haemolyticus*), Sa (*S. aureus*), Ec (*E. coli*).


FHP 2
FHP 11
FHP 15
FHP 3
FHP 21A
FHP 16
FHP 5
FHP 21B
FHP 18
FHP 7A
FHP 34
FHP 24

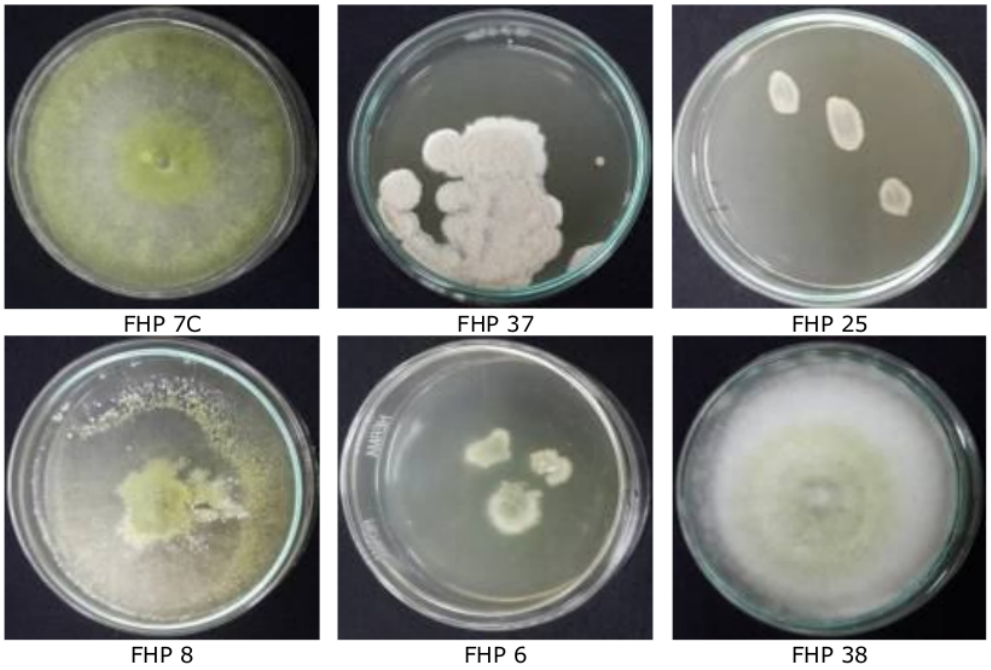


Figure 3. Morphology of potentially interesting fungi.

This study also revealed that FHP 3 and FHP 34 isolates show activity against 5 different MDR Gram-negative bacteria, namely *P. aeruginosa, E. aerogenes, S. haemolyticus, S. aureus* and *E. coli*. Among these, FHP 6 isolate was selected as the best candidate with the most potential as indicated by the clearest and largest inhibition zone (28.00±1.82) against the *E. cloacae* complex.

Antimicrobial resistance of bacteria is a growing worldwide problem (Okeke et al 2005). Nosocomial infections with MDR Gram-negative bacilli have considerable clinical and economic burdens. The MDR bacteria listed (Table 3) have emerged in many medical centres as particularly troublesome pathogens. The bacteria used as antibacterial indicator were divided into 3 groups, namely the Enterobacteriaceae (*K. pneumoniae, E. aerogenes, E. cloacae* complex, *E. coli*), nonfermenting Gram-negative bacteria (*P. aeruginosa, A. baumannii*) and Gram-positive bacteria (*S. haemolyticus, S. aureus*).

S. haemolyticus, commensal on human skin, are member of coagulase negative staphylococci bacteria causing septicemia, peritonitis, otitis, and urinary tract infections (UTC), as well as infections of the eye (Daniel et al 2014); Makki et al 2011). The threat of methicillin resistant *S. aureus* (MRSA) is increasing worldwide, mainly in Asia (Chen & Huang 2014). One of the most common bacterial infections in humans are the causative agents of multiple human infections (Tong et al 2015). In addition, previous studies mention penicillin as a determinant for the carriage of resistant *S. aureus* in two hospitals in Java (Lestari et al 2010). In the Semarang area, there are risk factors for nasopharyngeal carriage of *K. pneumoniae* and other Gram-negative bacteria that cause pneumonia and urinary tract infections in the ICU setting (Gaynes & Edwards 2005). *P. aeruginosa* is a frequent cause for respiratory problem, surgical site infection and urinary tract infection in patients from intensive care areas (Gaynes & Edwards 2005). Several studies reported that resistance rates increase to fluoroquinolones, cephalosporins and carbapenems, particularly among ICU isolates (Jones et al 2004; Streit et al 2004; NNIS System Report 2004).

A. baumannii is considered a hospital-acquired pathogen. It has been reported that carbapenem-resistant are feared for their potential to cause nosocomial outbreaks (Tacconelli & Magrini 2017). This pathogen is also recognized as an emerging pathogen in

many medical facilities and according to NNIS data, the proportion of infections due to *Acinetobacter* spp. has increased (Gaynes & Edwards 2005). *E. aerogenes* and *E. cloacea* complex are an important cause of health care-associated infections, with a notable propensity to acquire antibiotic resistance determinants (Mezzatesta et al 2012). Two species of this genus are most involved in healthcare-associated infections, namely Extended-Spectrum Beta-Lactamase ESBL and carbapenemase producers (Cabral et al 2017). The last is *E. coli*, a bacterium that is often used as a marker for faecal contamination of water, and the indicator microorganism in the World Health Organization (WHO) Tricycle project for antimicrobial resistance.

The previous studies mention that the use of marine fungi in this field has suffered neglect, despite them being extremely potent producers of secondary metabolites and bioactive substances (Kobayashi & Ishibashi 1993; Lang et al 2007; Raghukumar 2008; Yu et al 2008). Compounds with anti-microbial potential may be produced by marine invertebrate associated microorganism (Ayuningrum et al 2019; Cristianawati et al 2019; Sabdaningsih et al 2019; Kristiana et al 2020). A literature survey covering more than 23,000 bioactive microbial products, i.e. antifungal, antibacterial, antiviral, cytotoxic and immunosuppressive agents, shows that the producing organisms are mainly from the fungal kingdom. Hence, fungi represent one of the most promising sources of bioactive compounds (Brakhage et al 2004). Especially, the genus *Aspergillus* has been known to be a major contributor to the bioactive secondary metabolites of marine fungal origin. Particularly, antibacterial bisabolene-type sesquiterpenoids from sponge-derived fungus, *Aspergillus* sp. (Li et al 2012), cytotoxic hetero-spirocyclic γ-lactams from *A. sydowii* from sediment (Ren et al 2010), 14-membered macrolides from *A. ostianus* (Kito et al 2008), phenylalanine derivatives, and cytochalasins from the soft coral-derived fungus, *Aspergillus elegans* (Zheng et al 2013) and breviane spiroditerpenoid from the marine-derived fungus *Penicillium* sp. (Yang et al 2018). Therefore, fungi derived from marine sources are considered to represent a huge reservoir of secondary metabolites (Saleem et al 2007). The use of associated microorganism is very helpful due to the conservation issue. It is important to highlight anti-pathogen compounds that are produced by marine hard coral-associated with marine fungi in providing the possible role as an alternative drug.

The PCR product amplification. The method of visualizing the PCR products was staining of the amplified DNA product with a chemical dye namely ethidium bromide. It is indicating how much of a specific DNA or gene was presented in the sample. Agarose gel electrophoresis was used for visualizing and analyzing the PCR product. Out of 18 active fungal isolates, 14 isolates with proper DNA products were continued for further analysis. Results showed that the samples have a single band (n = 11), double bands (n = 2), and one sample was shown without band. This research was able to demonstrate the presence of amplicons ranged in 10 out of 14 samples tested, by the presence of a PCR-product band about 500-750 bp long, as seen on a 1% agarose gel with ethidium bromide (Figure 4). The first lane marked by (M) is the molecular marker, which is used to identify the size of the detected PCR product.

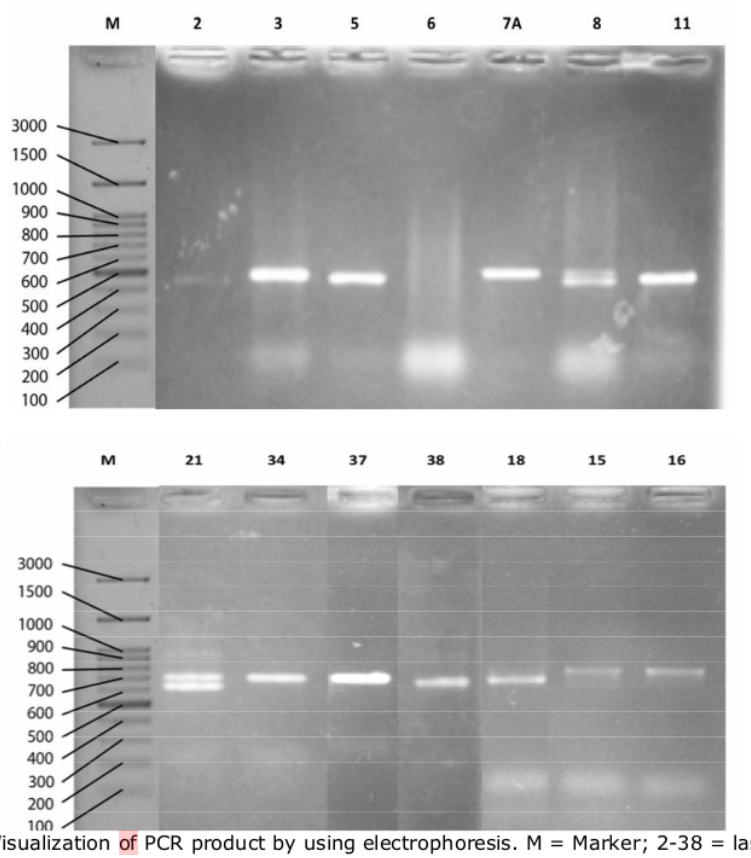


Figure 4. Visualization of PCR product by using electrophoresis. M = Marker; 2-38 = last number code of isolated fungi.

Conclusions. Marine fungi were successfully isolated from 8 marine hard corals. Out of 18 active isolates, the HCAF inhibited the growth of at least 1 pathogenic bacterium. Among these fungi, FHP-6 isolate was selected as the candidate with the anti-microbial potential as indicated by the clearest and largest inhibition zone (28.00±1.82) against MDR *E. cloacea* complex. FHP-3 and FHP-34 isolated from *Goniastrea* sp. and and *Acropora* sp. respectively have been recognized as the most potential antibacterial agent against severe MDR namely *P. aeruginosa, E. aerogenes, S. haemolyticus, S. haemolyticus*, *S. aureus*, *E. coli*

HCAF from Panjang Island, North Java Sea represent an untapped richness of an underutilised group of marine microorganisms and the possibility of environmentally friendly secondary metabolite producers with medical potential in particular against multiple MDR pathogenic bacteria. Further investigation of the structure of bioactive compounds from marine HCAF into the discovery of a compound with anti-MDR pathogen properties is needed, especially in bioassay guided purification.

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